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CONTENTS

No. 1 MARCH, 1931

HAROLD R. HAGAN. The embryogeny of the polyctenid, Hesperoctenes fumarius Westwood, with reference to viviparity in insects. One frontispiece and twelve plates (fifty-eight figures) 1

W. R. B. ROBERTSON. Chromosome studies. II. Synapsis in the Tettigidae, with special reference to the presynapsis split. Six plates (seventy-two figures) 119

ALFRED M. LUCAS. An investigation of the nervous system as a possible factor in the regulation of ciliary activity of the lamellibranch gill. Five text figures and four plates (twenty-one figures) 147

ALFRED M. LUCAS. The distribution of the branchial nerve in Mytilus edulis and its relation to the problem of nervous control of ciliary activity. One plate (eight figures) 195

KAGEYAS W. AMANO. The labyrinth of Bufo vulgaris Japonicus larva. Especially, upon the morphologic study of the perilymphatic space, in connection with the membranous labyrinth and auditory capsule. Ten plates (thirty-two figures) 207

CLEVELAND P. HICKMAN. The spermiogenesis of Succinea ovalis Say, with special reference to the components of the sperm. Two text figures and eight plates (seventy-eight figures) 243

GEORGE V. E. BROWN. The morphology of Spironympha, with a description of a new species from Reticulitermes hesperus Banks. Two heliotype plates (nine figures) .. 291

No. 2 JUNE, 1931

B. K. DAS. Observations on the 'renal-portal' perfusion in etherized birds. One figure. 309

H. E. JORDAN AND C. C. SPEIDEL. Blood formation in the African lungfish, under normal conditions and under conditions of prolonged estivation and recovery. Four plates (one hundred figures) 319

ORIHAY SHINJI. The evolutional significance of the chromosomes of Aphididae. One text figure and five heliotype plates (189 figures) 373

MILTON WALKER EDDY. Recovery from immersion in water. An index of metabolism and the condition of the gonads in Drosophila melanogaster Meigen and Popillia japonica Newm. Twelve figures 435

OLIN E. NELSEN. Life cycle, sex differentiation, and testis development in Melanoplus differentialis (Acrididae, Orthoptera). Two text figures and five plates (fifty figures) 467

RICHARD P. HALL AND ROSS F. NIGRELLI. The vacuome of the flagellate Chlamydomonas. Two text figures and one plate (eight figures) 527

FARRIS H. WOODS. History of the germ cells in Sphaerium striatinum (Lam.). Fifty-four figures 545

J. BRONTË GATENBY. Notes on the postnuclear, acrosome-seat granules, and 'vacuome' in Desmognathus fusca spermatogenesis. Fifteen figures 597

ELEANOR H. SLIFER. Insect development. II. Mitotic activity in the grasshopper embryo. Two figures 613

Hesperoctenes fumarius Westwood. Mature female with two large embryos, one of which is in position for extrusion. × 24. (Photograph by Doane and Hagan.)

THE EMBRYOGENY OF THE POLYCTENID, HESPEROCTENES FUMARIUS WESTWOOD, WITH REFERENCE TO VIVIPARITY IN INSECTS

HAROLD R. HAGAN

ONE FRONTISPIECE AND TWELVE PLATES (FIFTY-EIGHT FIGURES)

AUTHOR'S ABSTRACT

Four functional types of viviparity are recognized, and the last, pseudoplacento viviparity, is illustrated by a review of the embryogeny of a species of Polyctenidae. This insect normally has ten embryos in the reproductive tract in successive stages of development. The problem of fertilization is discussed, for there seems to be no spermatheca and spermatic clumps are present in the haemocoel. No organ of Berlese can be found. One, apparently a nymph, when sectioned revealed spermatozoa in even greater abundance than the mature females. Four to six of her offspring would seem to be paedogenetic.

Females liberate ova that are yolk-free, and no chorion is secreted about them. Blastomeres are distinct, the embryonic envelopes are formed as usual, and hemipteran embryology occurs. The trophserosa functions until blastokinesis takes place, when the pleuropodial extensions evaginate and encompass the embryo which now lies in a pleuropodial cavity. The pleuropodia function as nutrient organs, or pseudoplacenta, until shortly before birth. At birth the embryo is a little more than one-third the adult body length and bears strongly developed setae.

CONTENTS

Introduction 4
Acknowledgments 7
Material and methods 7
Historical review 9
Viviparity in insects 12
Preceding classifications 16
Proposed classification of viviparity 18
Discussion of the types of viviparity 19
Type 1. Ovo-viviparity 19
Type 2. Intussuetio-viviparity 22
Type 3. Exgenito-viviparity 24
Type 4. Pseudoplacento-viviparity 25
Reproduction in Hesperoctenes fumarius 25
Observations on the sexually mature female 25
The maternal abdomen 25
Female reproductive system 27
Discussion of the reproductive system 29
Impregnation of the female and the problem of fertilization 31
Discussion 31
The spermatozoa 31
Conclusion 36
Paedogenesis 38

Embryogeny 41
The ovum 41
Maturation of the ovum 43
Cleavage and formation of the blastoderm 44
Segmentation and the appearance of the extremities 47
Prerevolution stage 48
Blastokinesis 50
Postrevolution stage 51
The free embryo 52
Postrevolution stage, half-grown embryo 53
Precuticular stage 55
Cuticular stage 56
Orientation of the embryo 57
The nutrient organs 60
Follicular epithelium 60
Nurse cells 60
The embryonic membranes 64
Serosa 64
Amnion 64
Trophocytes 69
Pleuropodia 70
Germ-band stage 70
Early embryonic stage 71
Prerevolution stage 72
Postrevolution stage 74
Cuticular stage 78
Stage prior to hatching 79
Function of the pleuropodia 80
Nutrition and the migration of the embryo 85
Summary 87
Literature cited 90

INTRODUCTION

The production of living young by insects always has been a source of keen interest to entomologists. The class Insecta as a whole reproduces by eggs that are deposited in a suitable environment. This often proves to be a favorable stage in which to overwinter or to survive some other period of adverse conditions, or it may be simply an interval of apparent quiescence in the life history of the species. The appearance of viviparity, then, in species that are closely related to oviparous forms furnishes interesting exceptions to the general rule and striking examples of specific differences in the course of the reproductive process.

It is assumed that viviparity is a term simply expressing an incident in the ontogeny of an insect's development. Whether the egg hatches before leaving the parent's body or afterward is considered to be of little consequence. But the various ways in which the viviparous condition is expressed through physiological and physical adjustments on the part of parent or offspring have appealed to me as being matters of especial significance. It is the desire to bring out the importance of these changes rather than simply to stress the viviparous condition that has caused me to offer herein a new classification of the types of viviparity.

Among those embryos born alive with all of their variations in structure to fit the conditions of viviparous birth, only one has been recorded that supplies an example of viviparity involving organs that may be considered at all analogous to the mammalian placenta. This insect is Hemimerus talpoides Walker. Here the embryo derives its nourishment from the mother continuously as needed by means of a specialized development of the follicular epithelium.

In the present paper Hesperoctenes fumarius Westwood is presented as another example of this unique method of reproduction. The viviparous condition and its associated modifications, both in the mother and her offspring, are remarkable. None of the embryogeny of the family Polyctenidae has previously been described. Because of this, the embryological history is traced so far as my material permits. There is in this species an even greater specialization of the nutritional function in that the organs concerned have definite form and structure, whereas in Hemimerus the 'placental' organ is simply an amorphous mass of cellular material. They are so diversified and in one respect so unlike those of other insects that they have been discussed separately. New or rare variations in embryonic structures are included wherever they have been recognized. There has been made no attempt to compare the development of this species in its details with other species in general, for in its broader aspects it follows the plan of the hemipteran forms. Comparison,

however, is made with Hemimerus, one of the Dermaptera, which also has a number of specializations relating to the viviparous condition. Some interesting contrasts or similarities between the two are pointed out.

The first abdominal appendages of embryo insects were called pleuropodia by Wheeler ('90). To date many writers have drawn attention to them and their possible function. Here the attempt is made to explain their present function in Hesperoctenes fumarius Westwood as one of nutrition.

Many cases of viviparity present difficulties in the impregnation of the female and the fertilization of the egg. They apparently have arisen in the species whose embryonic history is given herein and some of their complexities have been presented.

The discovery of specimens of Polyctenidae is so infrequent that while viviparity in the family was announced by Jordan in 1911, no attempt has since been made to investigate the method of reproduction. Indeed, Jordan ('11 a) says in reference to them that they ". . . . appear to be very rare. The collection of the British Museum comprises only 27 specimens; but this number is very large in comparison, as less than a dozen specimens are mentioned as being contained in other collections. This rarity is not merely apparent, as in the case of so many other insects, but is real, we believe." The Stanford University collection has eighteen specimens, thirteen adults and five nymphs, representing altogether five species. Owing to the unfortunate necessity for the systematist to macerate and remove the internal structures in order to study the specimens, these show nothing relating to my paper. Professor Ferris, however, has stated that embryos were observed in some of the specimens before they were prepared for mounting.

In view of the above, no apology is due for the lack in my paper of some important steps in the development of this insect. The embryonic history is so extraordinary and the species so rarely found that it is inexcusable to refrain longer from presenting it. As it is, my material represents what

may be considered a very large collection of one species of Polyctenidae.

It might be well to recall to mind the lamentable fact that, in the fifty years or more that these insects have been known, no description of any of the internal anatomy of the adult has been given. The writer constantly has felt the need of earlier accounts as a guide to his own work for at least some of this material and has keenly sensed his responsibility in the study of the species.

ACKNOWLEDGMENTS

The exceedingly rare and interesting material for this paper as well as the direction of my interest to the study of pleuropodia was furnished by Prof. W. M. Wheeler, of Harvard University, to whom my sincere thanks, as those of so many students of insects, are due. The preparation of most of the slides and their preliminary study occurred at the Bussey Institution. Further acknowledgment is extended to Professor Wheeler for his kindness in reading the manuscript.

An expression of my appreciation also should be given to the members of the faculty of the Department of Zoölogy of Stanford University who have shown invariably a spirit of helpful encouragement when called upon. Professor Ferris, especially, with whom the work was done at Stanford, has taken unvarying interest in it and has offered constructive and kindly criticisms while it was in progress.

MATERIAL AND METHODS

The insects have been determined by Professor Ferris as Hesperoctenes fumarius Westwood, a species of Polyctenidae. This family belongs to the order Hemiptera and is closely related to Cimicidae. The specimens studied were collected from bats by Mr. Robert Gardner at Havana, Cuba, and sent to Prof. W. M. Wheeler about the year prior to my receiving them. Preservation was in 70 per cent alcohol, and the material in consequence was quite brittle and difficult to sec-

tion. Another feature adding to my difficulties was the heavy sclerotization of the older embryos and the resistance offered by the setae. The female, too, was abundantly supplied with both coarse and fine setae difficult to remove entirely.

Dissection was unsatisfactory under the existing conditions, as the tissues broke apart at the slightest touch. Prolonged immersion in water and in cedar oil alike failed to make dissection easier. After two unsuccessful attempts, no more specimens could be spared and gross dissection was discontinued in favor of sectioning.

It was necessary to use a combined paraffin-celloidin method modified to suit the material. Sections were cut 7 μ and 10 μ in thickness, but the former proved to be more satisfactory.

In staining, Ehrlich's haematoxylin and orange G were used in some cases, while borax carmine or Ehrlich's and eosin were found to be more satisfactory. Indeed, all of the tissues stained with orange G were run into the alcohols again later and restained in eosin. Eight gravid females were sectioned and four nymphs taken from the dissected females also were prepared. Two mature females were mounted entire, in order to refer to them when necessary to recall the general positions of the contained embryos, which are plainly discernible in the abdomen. A few young nymphs in alcohol were carefully locked up in a laboratory drawer, in order to examine them later to learn the fate of the pleuropodia after eclosion. A newly appointed assistant, in his efforts to clean up the room, pried open the drawer and threw out the specimens.

Drawings have been made by camera lucida in which most of the details have been filled in after the outlines and proportions were obtained with the aid of this instrument.

It should be pointed out that the lack of proper fixation of this material—a condition often unavoidable in collecting unexpected specimens—may have resulted in shrinkage and distortion. The staining procedure also, following alcoholic fixation, is notoriously unreliable.

With these things in mind, one should be constantly on guard against misinterpretations, and some details recorded must be subject to further confirmation and perhaps correction. Such inaccuracies, I hope, are reduced to the minimum.

HISTORICAL REVIEW

Literature relating to classifications of viviparity

The literature pertaining to viviparous forms is voluminous, but the attempts to classify the kinds of viviparity are few.

Holmgren ('04) subdivided his classification into two groups, the parthenogenetic and the amphigonous. In the first he included the species found in certain Hemiptera and the Cecidomyidae; in the second, those of the Neuroptera, Orthoptera, Coleoptera, Hemiptera (Coccidae), Diptera, Lepidoptera, and Strepsiptera.

The Diptera of his second class contain such divergent types of viviparity as viviparous Muscidae and the Pupipara.

This highly artificial arrangement apparently was followed very largely by Comstock ('24).

As the two classifications of viviparity are so similar, it may be well to bring out my objections to one of them, at least, under the general discussion of viviparity. I am convinced that both may well be revised in the light of the contributions made to the literature of viviparous forms. Further along in this paper I submit such a regrouping with some discussion of the functional aspects involved.

Literature relating to insects with placenta-like organs

The first species of insect whose embryological development shows this type of viviparity is Hemimerus talpoides Walker, an ectoparasite of the African rat Cricetomys gambianus Waterh. Some difficulty was experienced in finding its systematic position, but finally it was relegated to the order Dermaptera. Its viviparity was reported first by Hansen ('94). In preparing slide mounts of the insect for purposes of systematic study he was astonished at finding six

young in the ovarioles of one of the females. Concerning them he writes (pp. 78–79):

> The most remarkable feature is, however, that in the 4 greatest specimens, issues from the membrane between the head and the protergum an unpaired organ (b), slender at the basis but rapidly rather stout . . . .; to the best of my understanding this organ must be in connection with the interior wall of the female genital organs and thus serve the nutrition of the young ones. . . .

Again he says (p. 79):

> This curious propagation places the Hemimerus totally isolated from all known insects, as far as I know, for the propagation of the Pupiparous Diptera differs very much from this, and the other viviparous insects always bear several or many young at a time. . . .

That Hansen's observations, necessarily very limited because of his scanty material, were entirely correct has been amply demonstrated by the intensive studies of Heymons ('09, '12). His first paper gave a brief account of the appearance and development of the 'placental' organ which furnished nourishment to the embryo by means of a direct connection with the mother.

His second contribution gave more details of the nuchal organ and supplied additional information relating to the general embryological development of this insect. In Hemimerus the amnion and serosa are involved as part of the nutrient system together with the maternal anterior 'placental' mass. The anterior foetal 'placental' mass discharges its nutriment into the 'vesicula cephalica' through the amnion. A maternal posterior 'placental' mass plays a part, too, in the nourishment of the embryo, but one of lesser significance. Except for the loss of the chorion, the peculiar development of the pseudoplacenta with the attendant destruction and absorption of the follicular cells used in nourishing the embryo, the other embryonic history offers nothing of exceptional interest and is, as Heymons asserts, quite similar to Forficula in the same order of insects to which Hemimerus belongs.

It is to be regretted, however, that his material was not more abundant, that other details of interest, and still lacking, could have been presented.

Jordan ('09) gave a brief account of the anatomy of Hemimerus and its food. The morphological relations of the reproductive organs of both sexes and their location in the abdomen are discussed. A brief description of the position of the embryo and the nuchal organ in the ovarioles also appears.

Viviparity in the Polyctenidae—which is the second example of this kind of reproduction—was discovered by Jordan ('11 a) when mounting a few specimens in balsam for systematic study. His discovery was mentioned elsewhere during the year (Jordan, '11 b) without further details being given. In another paper Jordan ('12) refers to types of viviparity based upon fertilization phenomena and cites his discovery of the remains of an embryo in a species of Polyctenidae. All of the species subsequently studied in this family appear to be viviparous, therefore he maintains that it is a characteristic of the entire family. He recognizes that ovarial pregnancy must occur, since the development of the embryo takes place in the ovarioles. While he figures part of the outlines of an embryo as distinguished through the body walls of the mother, he limits his discussion largely to a consideration of the morphological distinctions between nymph and adult for systematic purposes and evidences of relative phylogenetic ages of the species. No attempt is made to trace any embryonic relationship to the mother. From his drawings there seem to be distinct differences in the relative size, shape, and position of the embryos in utero in the species figured, compared with Hesperoctenes fumarius Westwood.

Literature relating to pleuropodia

Inasmuch as this paper considers especially the importance of the pleuropodia in the economy of Hesperoctenes fumarius Westwood, it is desirable to call attention to the literature on these organs. Fortunately, the present knowledge of pleuropodia and speculations regarding these interesting abdominal appendages have carefully been summarized.

Extended treatment of this literature is, in consequence, unnecessary.

Graber's paper ('88) was a résumé of the existing literature on the pleuropodia. It was followed by Wheeler's paper ('90), bringing together the information on pleuropodia that appear to be rather well developed in the embryo. At this time there were three suggested functions tentatively assigned to the pleuropodia: they were thought to be respiratory, glandular, or sensory. His conclusion is that these organs probably serve in a glandular capacity. Further use of his conclusions is made in subsequent pages.

Very recently, the subject again has been taken up and very satisfactorily summarized by Hussey ('26). Following a short but detailed account of the appearance and growth of the pleuropodia in Belostoma flumineum Say and Ranatra fusca Palisot de Beauvois, she reviews completely the literature on the subject. Of the theories advanced regarding their function, she, too, favors the glandular hypothesis. The paper concludes with an extensive bibliography, to which the reader may be referred.

VIVIPARITY IN INSECTS

Viviparity is a term that has been accepted universally by entomologists to designate the birth of living young in some pre-adult stage other than the egg. This is in contrast to the word oviparity, which refers to the deposition of eggs that subsequently hatch into one of the pre-adult stages. This use of the term, while etymologically correct, refers simply to the physical fact of birth. It fails entirely to take into account the essential point to be considered in such a birth, that is, the morphological changes involved in particular kinds of viviparity. Such alterations point to profound physiological readjustments in the insect economy which must follow to permit the survival of the species under their influence.

Numerous examples of the birth of living young exist in groups in which almost no alteration of these parts has occurred. Still, in many species more or less complete reorgani-

zation of the reproductive structures has undoubtedly been accompanied by the assumption of diverse and complex physiological developments. These changes in the reproductive organs of insects range from a condition of no apparent divergence from the oviparous type of structure through many variations reaching finally to conditions closely approaching true placentation, intra-uterine incubation, and the almost complete abolition of yolk storage by means of follicular cells.

As time passed and more examples of viviparous birth were found it became evident that this phenomenon had arisen sporadically many times in the insects, several orders being involved. From the phylogenetic standpoint viviparity establishes no generalizations for the systematist except in a limited sense. For instance, it may be assumed that the phenomenon arose earlier than the existing species of Hippoboscidae among the Diptera. Even this, however, is no great assurance of affinity in itself, for the type of viviparity exhibited by Hippoboscidae is duplicated in other families, as in the oviparous Muscidae, where the genus Glossina reproduces viviparously in a manner quite similar to the former family. On the other hand, it is quite unlike other viviparous genera of its own family in several essential points.

Viviparity was the method of reproduction in the ancestors of the order Strepsiptera, for, according to Pierce ('18), it is an ordinal characteristic, and the only order showing such specialization throughout its species. In addition, it is thought that viviparity is a family characteristic in all species of Nycteribiidae, Streblidae, and Polyctenidae. Elsewhere among the insects it appears occasionally, so that the total number of species showing viviparity is surprisingly large, Hemiptera and Diptera having the bulk of these species.

The fact that an insect deposits its young in the form of larvae or eggs is a point of secondary importance. In other words, the hatching of the egg within the maternal uterus or after deposition is simply a physical phenomenon of slight significance unless it be due to structural changes or physio-

logical readjustments on the part of mother or offspring, or both. In succeeding pages examples are given of cases of accidental viviparity that may be held to have a bearing on the question of the importance of viviparity.

In the case of viviparous species whose embryos require a constant and relatively high temperature, as, for example, in the mammals, uterine incubation has an important bearing because of the thermal factor. This is not true for insects, as they have a relatively wide latitude in this matter. The body temperature of the mother is approximately that of its surroundings. It cannot give the offspring any advantage so far as this factor is concerned and the latter could develop as well in another situation.

The mother might secure an advantage in placing her offspring in a favorable situation for feeding if it be hatched, whereas an egg would not do. Certain examples of parasitism often are cited in support of this concept, but I believe numerous cases of egg deposition in other species and its universality in Mallophaga, Anoplura, and Siphonaptera throw considerable doubt on the validity of this contention.

Some external parasites must derive an advantage in the production of a few young over a long period of time, for the wasteful extravagance of yolk storage in many eggs is thus eliminated. The death rate, too, probably is lowered. This is especially true if the parasite lives constantly upon the host and takes food that is readily assimilable and always available. Just how far this advantage is counterbalanced by the additional drain upon the mother's body because of the necessity of transporting to her offspring the nutriment required for the larval life of the latter is unknown. It might, conceivably, be a disadvantage, for the larva must endure all of the hazards suffered by the mother.

One might even assume that viviparity in Polyctenidae is an important limiting factor in their population and dispersal. Coupled with this we must include their eyeless and flightless condition. If viviparity were an advantage to them, we would expect to find them widely distributed on their hosts

and occurring in numbers comparable to Anoplura and Mallophaga which are oviparous. This is not the case, and it was mentioned earlier that they are quite rare.

Viviparity also appears to accompany no fixed plan of organization in the insects as it does in the Mammalia. That is, no one type of reproductive system, method of nourishment, or other factor holds for all of the forms bearing living young. On the contrary, most viviparous species exhibit little if any variation in their reproductive systems from related oviparous species. In consequence, their variations in viviparity follow phylogenetic limitations rather than functional convergences. These differences, however, do seem to be somewhat limited in number of types while unlimited in the degree of expression of any one type, as will appear below. Perhaps it is because other changes, that may have arisen in the past along with viviparity, have in many cases necessitated modifications so great in development on the part of the embryo or in the mother's own metabolism or morphology that the adaptability of the species concerned was not capable of making the adjustments and extinction followed. Or, indeed, the appearance of viviparity or changes leading to it might have been too abrupt and the species thus was eliminated. In spite of the seeming diversity and complexity now known to be present in the different methods of viviparous reproduction of insects, these cases seem to me to fit into a certain grouping of relatively few types.

It is possible that some of the forms now seasonally viviparous are so because of environmental conditions quite similar in kind and yet more constant in effect than those which affect insects that are only occasionally viviparous. Occasionally viviparity may result, then, from occasional stimulation. Seasonally viviparous species might easily become permanently so if the controlling factors were constantly in force. The tendency to occasional viviparity may be of significance in revealing species whose normal method of reproduction is perhaps in a state of flux, of shifting from one form of reproduction to another under the influence of ecological

factors as selection agents. At least these species are more plastic than those that cannot so accommodate themselves when stimulated to do so.

More cannot be said in this paper on a subject which has ramifications extending into several aspects of insect economy and upon which many have preceded me.

Preceding classifications

That the study of the production of living young merits greater discrimination in the employment of terms connected with it has been clearly recognized. Hansen ('94, p. 80) specifically called attention to this when comparing reproduction in Hemimerus with that in certain cockroaches. He says of the latter that they show ". . . . sufficiently that the development of these forms is essentially different, presenting but a point of resemblance of secondary nature in the birth of living young."

One of the most complete summaries of our present knowledge concerning viviparity was made by Holmgren ('04). He disregarded entirely, however, the essential physiological aspect of the question and treated viviparity as simply the physical production of living young. He realized that such phenomena were not all alike in origin, for "Wir können somit vivipare Insecten mit parthenogenetischer Entwicklung und vivipare Insecten mit normaler, amphigenetischer Entwicklung unterscheiden" (p. 431). Yet these categories do not prove to be fundamental after all, as in every case insects arise from eggs (Comstock, '24). The fertilization process has the rôle in nature of initiating development and providing genetic variation, or the male element may be in many cases entirely unnecessary and wanting. It may certainly have other functions as well, but I doubt if the union of the male germ cell has any influence over the type of intra-uterine life of the developing offspring. Its significance lies entirely aside from the embryonic development of the progeny.

Keilin ('16, pp. 393–394), discussing viviparity in his excellent summary of this subject in the Diptera, immediately dis-

carded certain cases of occasional viviparity and incorporated into his discussion only those that he classified as constantly viviparous. These he divided into two major groups, as follows:

I. Ceux qui incubent seulement pendant la phase embryonnaire, c'est-à-dire dont les larves ne sont jamais nourries dans l'utérus. Ce sont plutôt des ovo-vivipares ou des ovo-larvipares pondant des oeufs mûrs ou les larves qui viennent d'éclore.

II. Ceux qui, outre la phase embryonnaire, incubent encore la larvae; c'est-à-dire les formes chez lesquelles les larves écloses sont nourries dans l'utérus maternal grâce à certaines dispositions spéciales.

For the purposes in mind in his discussion of viviparity Keilin eliminated from his consideration those species that are only occasionally viviparous. To dismiss them from this paper would appear to be a mistake in arranging a grouping of kinds of viviparity. My endeavor is to include in a classification of viviparity all of those forms that we know are capable of giving birth to living young.

Comstock ('24) divides the types of viviparity into two kinds (p. 192): 'Viviparity with parthenogenetic reproduction' and 'Viviparity with sexual reproduction.' One might be justified in assuming from this phrasing that these two divisions differ materially in that one is asexual while the other is the sexual process, but obviously he does not intend to convey such a meaning. On the preceding page he states that all insects are developed from eggs and he further declares that all eggs are germ cells. Reproduction by means of germ cells, whether parthenogenetic ova or fertilized gametes, is a sexual process and is in contradistinction to somatic reproduction as budding and fission, which are asexual types.

The subdivisions under his first class of viviparity are founded upon the state of physical immaturity of the parent. This class includes: paedogenetic larvae, paedogenetic pupae (citing Chironomus grimmii, which he says, deposits eggs!), and viviparous adult agamic females (aphids). His second

group is subdivided on the degree of immaturity of the offspring at birth and includes sexual viviparous insects giving birth to nymphs or to larvae and sexual viviparous insects giving birth to old larvae.

The above classification separates into one group the orders Strepsiptera and Orthoptera, for example, because their eggs are fertilized. The entire developmental history of the Strepsiptera is a series of peculiar adjustments to the viviparous condition totally different from any of the viviparous Neuroptera, Orthoptera, Coleoptera, etc., with which the order is allied in his grouping. The Orthoptera, for example, much more closely follow the nutritional history and early embryonic life of Cecidomyidae. The last family, nevertheless, is in the other group with development from unfertilized eggs.

Since the previous classifications of viviparity apparently are based upon the presence or absence of the fertilization process, I believe them to be unsuitable in giving expression to the true types of embryonic history involved. Inasmuch as the ontogeny of an individual properly begins with the development of the egg into an embryo, it appears to me that what happens to the egg prior to the initiation of the cleavage stages is of relatively minor importance compared to the sequence of events that follow. For this reason I have disregarded the matter of the parthenogenetic or fertilized ovum in bringing together the types of development undergone by the embryos.

Proposed classification of viviparity

Sufficient evidence now has accumulated to warrant an attempt to reduce the known cases of the production of living young to a classification in which a few simple characteristics will serve to distinguish between them. The divisions proposed below will emphasize the functional phase of the subject.

1. Ovo-viviparity. That type of viviparity in which the egg contains sufficient yolk to nourish the embryo until hatching. The larva is extruded by the mother without receiving additional nutriment

from specialized organs. Examples: Coccidae; Coleoptera; Sarcophagidae, etc.

2. Intussuctio-viviparity. That type of viviparity in which the egg contains sufficient yolk to nourish the embryo until hatching. The larva is retained in the maternal uterus and nourished by means of specialized organs. Examples: Glossina, Muscidae; Hippoboscidae; (Diptera).

3. Exgenito-viviparity. That type of viviparity in which the embryo, in a stage of development corresponding to the egg stage of ovo-viviparous forms, obtains its nourishment directly from the maternal tissues by means of a trophamnion, trophserosa, or trophchorion. Development occurs in the maternal haemocoel, not in the genital tract. Example: Strepsiptera.

4. Pseudoplacento-viviparity. That type of viviparity in which the embryo, until its extrusion, receives from the mother a constant supply of nutriment by means of placental-like organs. Examples: Hemimerus (Dermaptera); Hesperoctenes (Hemiptera).

Discussion of the types of viviparity

Type 1. Ovo-viviparity. In Sarcophaga carnaria Linné, according to Holmgren ('04), we find that the eggs pass from the ovaries into a vaginal pouch, where embryonic development takes place. The egg is fertilized some time in its descent in the reproductive tract. All of the embryogeny seems to be typical of the Diptera, as the egg contains the yolk required to bring the embryo to the point where it may begin an independent existence. At this time the eggshell is ruptured and the hatched larva is deposited by the mother upon a favorable food supply. No new structures are developed by the mother to supply further nutriment to her offspring while within the brood pouch. Some possibilities of physical adjustment in the mother, however, have been distinguished, for her vagina has proved to be capable of great distention. This fact permits the retention of the ova which, otherwise, she might be obliged to deposit at once. No evidence is available to show that this vaginal distensibility developed in order to permit egg retention. Perhaps it is more logical to assume that retention of the eggs resulted from unknown factors and their lodgment in the brood pouch occurred because of its capacity to store them.

This example may be used to illustrate the first type of viviparity which is proposed. The class contains the majority of the viviparous insects, so far as known. In it we see that the oviparous type of egg simply is retained until hatching finally releases the young, when the latter proceeds with its independent existence.

While Keilin discards the occasional viviparous forms and treats only those that are invariably so, I wish to include them in the above group with this limitation: By occasionally viviparous form is meant an insect that, being usually oviparous during its reproductive period, sometimes gives birth to living young after they have hatched from the eggs within the reproductive organs. This condition may come about under natural chemical, physiological, or physical stimuli, either extraneous or intrinsic in origin. It does not apply to the production of living young from eggs of an oviparous species that are retained in the reproductive system of the mother because of her premature death or injury prior to their deposition.

Living protoplasm is a plastic substance capable of considerable adjustment within its limits of possibilities. Often it responds to stimuli in remarkable ways. One of these I believe to be expressed by the viviparous condition in the above type of ovo-viviparous reproduction. Insect species may vary in their susceptibility to stimuli and the proneness of some one system of the body to react to them—as the reproductive system of certain occasionally viviparous species —may bear no relation to the rest of the systems in their behavior.

Entomologists frequently treat their specimens with chemicals and succeed in getting premature egg deposition. The writer also has witnessed larviparous abortion in Nycteribiidae and Hippoboscidae when the females have been dropped into vials of alcohol.

An illustration of such reaction resulting from climatological factors is gathered from the work of Roubaud ('09) and of Lloyd ('12) regarding abortions in Glossina. The former

states that while the unfertilized female may retain her ova in the oviduct, she also has the capacity of causing premature deposition of the larva in the case of the gravid female. Both conditions seem to depend upon the reaction of the nervous system to stimuli; the stimulus of copulation resulting in the descent of an ovum, for example, while excessive temperature may cause the prompt deposition of the immature larva. Lloyd writes, "Abortions were most numerous during September and October, while in the cooler months there were not so many."

Since this muscid reacts to the external influence of temperature to the extent shown above, it serves to emphasize the fact that it would not be difficult for stimuli to produce egg retention in susceptible species. This is simply the reverse of abortion and may very well be the normal reaction of certain species to changes in the environmental conditions at present surrounding them. Of this we have no proof, but the potential possibility undoubtedly is present.

Egg retention, or delayed deposition, as one prefers, accounts for most cases of occasional viviparity among flies. The environment may vary considerably, but, if within the critical limitations of the species, the eggs develop their embryos and hatching follows. We see this frequently when eggs of insects are collected and stored until hatching takes place. Or, as frequently happens, gravid females are captured by the entomologist and killed, and the eggs within the reproductive system of the insect complete their development later and the young appear. The different situations in which the eggs were lying in the two cases evidently had no influence on their ontogeny. An example of the difficulty found in distinguishing between occasional ovo-viviparity and oviparity is shown by a note that may be quoted from the Transactions of the Entomological Society of London, p. lxiv, 1911: "Dr. Chapman remarked upon certain supposed instances of viviparous butterflies, saying that the idea originated in a mistake, the eggs having been ready to be laid at the time of the parents' death."

The possible evolutionary course of this development toward viviparity might briefly be illustrated by an example from the order Orthoptera. This case, however, may result from stimuli arising within the species involved rather than in the action of extraneous influences. The limiting factors may be physiological or genetic in nature, or both.

Many of the Orthoptera secrete oothecae in which the eggs are placed. The females usually abandon them as soon as oviposition is completed. The family Blattidae, or cockroaches, behave differently, as Holmgren ('04) already has pointed out. Species of the genus Blatta commonly carry the ootheca about with them, protruding from the abdomen. It is dropped some time before the young have hatched. Blabera, Eustegaster, and Oxyhaloa retain the ootheca within the vagina while the offspring develop. Riley ('91) found that the retained ootheca in Panchlora is much reduced, as he

This egg capsule is practically wanting in *Panchlora viridis* and can be detected only by very carefully dissecting the egg cluster, when a thin membranous sheath is found to enclose the inner or basal half of the mass or about one-half the length of the eggs. Judging from this rudimentary nature of the egg capsule, if this partially inclosing membrane may be so styled, the colleterial glands must be much reduced or almost wanting in *Panchlora*.

With these considerations in mind, I have been led to the conclusion that occasionally viviparous species perhaps have parts of the reproductive system in such an unstable state that their oviparous condition may readily be replaced by ovo-viviparity.

Type 2. Intussuctio-viviparity. Quite a different case may be presented where the larva is not extruded to shift for itself until it is ready for pupation. There is here a very definite utilization of glandular secretions by the larva, and its continued nourishment, after hatching, accompanies maternal incubation. This is distinctly an advance in specialization, for in this type of viviparity the entire embryonic history up to pupation is dependent upon the mother. Even

the pupa stage in certain cases may be passed in situations selected by the mother, for Scott ('17) relates that Muir has observed a species of bat fly, Eremoctenia progressa (Muir), which in pressing its larva to the body of the host causes the offspring to adhere there during pupation. He cites also the work of Rodhain and Bequaert to show that Cyclopodia greeffi Karsch. even leaves the host to seek a sheltered spot in which to leave her larva. She presses it down tightly, where it remains during the pupal period.

Viviparity in the tsetse-fly (Glossina) was discovered by Bruce ('97) in his early researches on this genus in the years 1895 and 1896. Since this time several species have been described as viviparous by various authors. Minchin ('05), Stuhlmann ('07), and Roubaud ('09), especially, have treated in more or less detail the histology and nutritive relations existing between mother and larva during the latter's growth within its parent. Excellent summaries of the anatomy of the female genital tract have been given by Austen and Hegh ('22), Newstead, Evans, and Potts ('24).

The egg in passing through the oviduct is fertilized, presumably just before entering the uterus, as the spermathecal ducts open into the oviduct at this point. Embryonic development is completed by the aid of the contained egg yolk, but upon hatching, the larva derives its nourishment from the secretions of the 'milk glands' which branch profusely throughout the abdomen and open into the uterus. Referring to the function of the uterine glands, Minchin (p. 544) says: "There can be no doubt that these glands serve for the nourishment of the larva in the uterus." He describes them as being few in number and slender in the virgin female, while the gravid female contains numerous tubules which practically fill the abdominal cavity, each tubule in the latter instance becoming much thicker in diameter.

Roubaud states that the secretion from the uterine glands is absent when no larva is in the uterus; also, the secretion does not simply flow into the uterus, but the larva must actively engage in sucking its nutriment from these glands

whose ducts terminate in a papilla immediately in front of the larval mouth. The larva passes through three instars, separated by two molts, during its intra-uterine life. After deposition, it feeds no more, but quickly pupates.

Other insects having similar developmental histories are the Hippoboscidae and presumably Nycteribiidae and Streblidae.

Type 3. Exgenito-viviparity. The third type of viviparity is illustrated by the peculiar development found in all of the order Strepsiptera. Brues ('03), working with Acroschismus wheeleri Pierce (Xenos peckii Kirby), found that this strepsipteran nourished its embryos in part by means of a trophchorion developed very early in embryonic life. The nourishment is derived from the fat-body of the mother. In this connection Hoffman ('14), describing development in another species, says:

> Es dürfte wohl ausser Zweifel, dass der Fettkörper neben dem Eidotter bei der Ernährung der Embryonen eine hervorragende Rolle spielt: Im Laufe der Embryonalentwicklung erleidet er langsam eine Degeneration, die in einer allgemeinen Verminderung seiner Substanz und dem Auftreten zahlreichen Vacuolen seinen Ausdruck findet.

Hoffman believes, however, that the absorptive structure consists of a peculiarly developed two-layered 'chorion,' one layer of which is cellular in nature. Without commenting upon this phase of the question, it is desirable only to point out that the ova become embedded in the fat-body and there absorb the material stored in the latter while undergoing part of their embryonic development. Brues finds that all of the ova develop simultaneously, while Hoffman ('13, p. 393) and Pierce ('18, p. 402) assert that several stages of embryonic history may be secured from one female in the species that they observed. None of the offspring in this order escape from the mother by means of oviducts and genital openings. This, too, is unusual in insects, although cecidomyid paedogenetic larvae escape from the haemocoel after devouring the mother's internal anatomy. Because of these dif-

ferences between strepsipteran development and that of the ovo-viviparous type, the former cannot be included therein. As the larvae of the Strepsiptera act in the capacity of destructive parasites within the maternal body, they cannot be included with the remaining groups either, for by contrast the latter produce offspring that succeed one another in time and there are definite structures to ensure their nutriment as larvae or embryos without injury to the mother or active destruction of tissues. It is recognized that embryo insects always develop at the expense of the mother and therefore may be considered as parasitic. With this viewpoint in mind, the strepsipteran embryos might be classed as destructive parasites, while the embryos of the two remaining groups with nutrient organs may be designated as benign parasites. Therefore it has been considered necessary to segregate them into a separate and very distinct type of viviparity.

Type 4. Pseudoplacento-viviparity. The last type of viviparity is characterized by the presence of specialized structures that continually supply the embryo throughout its uterine life with nutriment derived directly from the mother. The nearest approach to this condition is in group 2, but these two types differ in that group 2 has an ample supply of yolk deposited within the egg which nourishes the embryo until hatching, while group 4 has ova that are yolk-poor. The present paper is devoted to illustrating this type of viviparity in Hesperoctenes fumarius Westwood.

REPRODUCTION IN HESPEROCTENES FUMARIUS

Observations on the sexually mature female

The maternal abdomen. The following brief account is an attempt to visualize the disposition of the more important organs in the abdomen of the female Hesperoctenes. It is presented with the function of reproduction in mind, and only those structures affected by the viviparous condition are discussed. In this manner alone can we realize how important a place the pseudoplacental type of reproduction has assumed in the insect economy where almost the entire

maternal abdomen is given over to the incubation and nourishment of the offspring.

In figure 1 is shown the internal structure of a female nymph that presumably is molting the last nymphal exoskeleton before becoming a mature insect. The central part of the abdomen is filled almost entirely with the alimentary system, of which the major portion is mesenteron. It connects anteriorly with the oesophagus, and posteriorly it opens into the intestine. Except for these attachments, the mesenteron, absorptive in function and continually filled with food, lies free in the surrounding blood space, or haemocoel. Just under the ectoderm the parietal fat-body cells are found in an almost unbroken sheet. These serve as storage cells for the surplus nutriment. There is no visceral fat-body. The intestinal portion of the system with the malphigian tubules is crowded into a posterior and dorsal position.

In the nymphal stage the epithelial cells of the mesenteron have become tall columnar and have assumed the same shape as the adult's midintestinal cells (fig. 40). The cytoplasm is glandular at the tips, and occasionally the cells discharge their products into the lumen of the tract. Whether the cytoplasm merely thins out and ruptures or whether the entire distal portion is pinched off is not known. The entire cell is coarsely granular and stains deeply. The cell margins are poorly defined and appear ragged under the microscope. The nuclei of the digestive epithelium are large, elongate, and each contains a large nucleolus. Chromomeres also are present. The nuclei are disposed with their long axes perpendicular to the basement membrane. Between the cell bases are found single cells which I assume are replacing cells. A thin basement membrane surrounds them, and ectally is a single layer of epithelium, very thin, with small and widely scattered nuclei. No muscular layers could be found covering the mesenteron.

The reproductive tract is the next important system, judging by the space devoted to it. Its size and location in the body are shown in figure 2, which is a longitudinal section a

little to the right of the middle of the nymph. The paired oviducts are found extending along the sides of the mesenteron, and their ovarioles project above and forward to the thoracic region. They are the first to be bathed in the blood, laden with nutriment, as it comes directly from the absorptive surfaces of the digestive tract. Thus the embryos within may be assured of a constant and rich supply of food.

The abdominal musculature is inconspicuous, consisting of thin tissues immediately under the hypoderm. The thoracic musculature is crowded dorsally and the nervous system is compressed ventrally into the smallest possible compass. In spite of this concentration, however, both of these tissues occupy considerable space.

The condition in the matured female is quite different, as one sees in figures 3 and 4. These sketches serve principally to indicate the altered condition of the reproductive tract when filled with embryos in varying stages of development. Figure 3 is a sectional view a little caudad to the middle of the abdomen. The embryos here have crowded the intestine against the fat-body and ectodermal wall. In the second outline the longitudinal view of the maternal abdomen is filled with a chitinized embryo almost ready for birth. A second embryo, slightly over half-grown, is not shown in this section, but is present beside the anterior third of the embryo shown. The alimentary tract is obliged to take a devious course through the abdominal region during the entire reproductive life of the insect. Its direction and position alter as the embryos grow and migrate down the oviducts.

Female reproductive system. The ovaries are paired. Each apparently consists of two ovarioles which are entirely separated from one another. Posteriorly, the ovarioles unite with the paired oviducts, which in turn fuse to form the common oviduct. The paired oviducts are much longer than the latter and are thin-walled, consisting of a single layer of squamous epithelial cells with flattened and widely spaced nuclei. The external opening of the common oviduct is concealed by the last visible sternite.

Great difficulty was experienced in tracing the course of the tubules. From the point of attachment near the anterior margin of the first abdominal segment they run forward into the mesothoracic and metathoracic segments, thence ventrally and posteriorly. In addition, their lateral displacement is subject to great fluctuation, due to the variation in size of the contained embryos and the migration of the latter down their respective ovarioles and oviducts.

The terminal filament is an extension of the tunica propria surrounding the germarium. It apparently is not cellular, but appears longitudinally striated, as though made up of fibrous substance. Although an outer layer of tissue could not be distinguished investing the filament, there were a few very small chromatin bodies scattered infrequently over the surface, indicating that a thin, poorly staining tissue layer was in contact with it. The outer surface of the filament showed a distinct lamella, and at the end of the germarium there seemed to be a very definite separation between the end chamber and the enveloping tunica propria. The inner lamella, if present at this point, was not visible in my sections. Anteriorly, the terminal filaments seem to be attached rather near together to the dorsal diaphragm. Posteriorly, the terminal filament forms a thin protecting sheet over the germarium. There is but little increase in the diameter of the filament at this point, the germarium simply extending into it as a central core of cellular tissue.

The germarium is about three times longer than broad, the measurements in one case being 105 μ long and 40 μ wide, but these dimensions vary in the individual germarium, depending upon the extent of its expansion by the contained ovum. The cells within it at the upper end appear to be rather densely packed, so that their greatest diameter is at right angles to the length of the germarium (fig. 5). Lower the cells are quadrate and the large rounded nuclei are subcentrally located, being nearer the cell bases. The chromomeres in the nuclei are prominent and a linin is present. No mitotic figures are to be seen in this layer of germ cells. The ger-

marium in longitudinal section is shown in figure 6, and figure 7 gives an enlarged view of a few of the germ cells.

The nurse cells are situated posteriorly to the germ cells as an epithelial layer of the tubule. The merging of the germarium into the vitellarium presumably is below the nurse cells, but it is so gradual as to pass unnoticed. On the other hand, the boundary between the germ cells and the nurse cell area is readily distinguishable by the difference in the appearance of the cells of the two regions. The nurse cells are not so regular in outline, are densely stained because of the granular inclusions, and the nucleoli are very large. They are shown in figures 6 and 8. They do not appear to occupy the lumen of the tubule at this point, but their secretions apparently do. This the ova must pass through in their descent. The question of the origin and probable function of the nurse cell mass derived from this region and following the egg in the latter's migration down the oviduct is discussed under the subject of nutrient organs.

The germarium opens into the larger and elongate vitellarium. Ectally, the vitellarium seems to be covered by a very thin epithelial-tissue layer with small flattened nuclei. Within this no tunica propria could be demonstrated, but entally a single layer of epithelium lines the tubule. The cytoplasm of the epithelium is finely granular, with an alveolar appearance near its free inner surface. No cell walls were visible, but cell boundaries are distinguishable by the increased number of granular secretions at their borders (fig. 9). The nuclei of these cells are very large, ovoid, and flattened in a plane parallel to the cell layer (fig. 10). Their chromatin granules are numerous and small, but the nucleoli are prominent, staining deeply.

Discussion of the reproductive system. Jordan ('12, p. 344) came to the conclusion that "There are apparently very few ovarial tubes (two or three on each side) and each contains only one embryo, as in the case of *Hemimerus*." His estimate of the number of tubules is quite correct, two pairs being all that can positively be determined from my slides.

The tubules are frequently so displaced that they sometimes appear to be very widely separated from one another. His statement that only one embryo occurs in each ovarial tube is probably incorrect and results perhaps from the macerated condition of the material examined. Younger embryos would be destroyed or made unrecognizable by the desiccation to which they were subjected in his specimens. An examination of the gravid females shows that, on the average, they contain in their ovarioles or oviducts offspring in various stages of development as follows: one oocyte, one mature ovum, one embryo in early blastoderm formation, one embryo beginning segmentation, and six older embryos.

This tabulation demonstrates the fact that ten offspring may be present at any one time in this insect. If, in addition, one embryo has just been born and if another oocyte had been released, it might have been possible to have had a maximum of twelve embryos or ova present. Three of these would come from each ovariole, but the oldest embryos would be in the oviducts or the lower portion of the vitellarium. Probably there never are more than ten embryos and eggs present at any one time.

The germarium differs materially from that of aphids as described by Uichanco ('24) and of Pentatoma nigricorne described by Köhler and summarized by Schröder ('28). In the latter case the germ cells form a very definite zone of cells in the germarium posterior to a much larger mass of potential nurse cells. These nurse cells separate the germ cells from the terminal filament by a considerable interval of space. Immediately posterior to the germ cells are the follicular cells in a third distinct zone. Uichanco figures and describes the condition in parthenogenetic forms of Macrosiphum rosae. Here the germ cells appear posterior to the filament. They are in a resting-cell stage anteriorly, but the posterior cells can be seen probably entering the first maturation stage. Behind them is the 'formative egg follicle' through which each oocyte as discharged must pass. The germarium is greatly distended with the few but enlarged oogonia, while the vitellarium is strongly constricted behind.

Hemimerus ovarioles have rounded epithelial-like nuclei in their short, conical, terminal filaments. These are followed by a few transversely placed cells which separate the former from the germarium. The germ cells are enormous and alternate with almost equally enlarged nurse cells.

In Hesperoctenes fumarius the germ cells have little cytoplasm and their nuclei are very large. They lie adjacent to the terminal filament and enclosed exteriorly by its posterior prolongation. They were not seen to be undergoing mitoses until discharged into the vitellarium. The nurse cells lie in much the same position as described by Uichanco for the formative egg follicle. True follicle cells seem not to be present unless the cells now functioning as nurse cells originally were egg follicle cells in nature. This subject is given more consideration later.

Impregnation of the female and the problem of fertilization. Discussion. Insects reveal remarkable diversities in the manner in which fertilization of the eggs is brought about. The micropyle in the majority of cases is thought to permit the entrance of the male gamete into the ovum immediately prior to deposition. However, other instances are frequent in which the spermatozoa must proceed far into the reproductive system to penetrate the ovum before the chorion is secreted around the egg. The first type of fertilization might be termed gonochaetal pregnancy, while the second may properly be termed ovarial pregnancy (Heymons, '09). Ovarial pregnancy must occur in those cases wherein embryonic development takes place within the ovary.

Fertilization is insured in a variety of ways among Hemiptera. The spermatozoa may be retained in the seminal receptacle until the ova pass into the common oviduct, where union of these elements then takes place. In the aphids this occurs in amphigonous forms, while the seminal receptacle is wanting in the parthenogenetic generations of the same species and in this case no fertilization occurs. The scale insect, Icerya purchasi, appeared to be parthenogenetic, but Hughes-Schrader ('25) recently has shown this insect to be a pro-

tandrous hermaphrodite. Patton and Cragg ('13) call attention to the remarkable condition existing in Cimex, where the organ of Ribaga and the organ of Berlese permit the escape of the spermatozoa into the haemocoel. Here some eventually fulfill their function by causing ovarial pregnancy, while the remainder, being absorbed within the body cavity, serve perhaps as nutriment for the female.

Another consideration is the length of the ovulation period. In insects that mature their eggs simultaneously, one mating usually is sufficient to insure fertilization of all of the developing eggs. Where ovulation occurs continuously, a single mating often will not suffice to provide indefinitely an adequate supply of spermatozoa and copulation occurs at subsequent periods. The order Hymenoptera furnishes a striking and familiar exception to this in the honeybee. The queen mates only once in her lifetime, but she has an elaborate receptacle for the care and nourishment of the spermatozoa for a number of years. In general, it may be stated that any unusual condition as continual ovulation, ovulation in cycles extending over several seasons or the viviparous condition often will be found to have in association different and more or less specialized types of fertilization processes.

Fertilization takes place in Hesperoctenes fumarius in the anterior portion of the vitellarium about the time the ripe ovum reaches the stage of development shown in figure 14. Ovarial pregnancy, then, may be said to be the rule. Prompt beginning of embryonic development shows this to be true.

Ovulation is continuous, an ovum being found in one or two tubules at any time.

Because of the peculiar development of the serosa and the pleuropodia into nourishing structures, to be shown elsewhere in this paper, it would appear that spermatozoa would have difficulty in passing the embryos in the common and paired oviducts. The tendency on the part of the embryos probably would be to convert into nutrient material any spermatozoa attempting to pass them. It is possible, however, that this is not a serious bar to the progress of the spermato-

zoa, for they may be immune to the activity of these nutrient organs. Also, in later stages of embryonic development the pleuropodia seem to be more active in reducing their own bulk and extensions which surround the embryo and may thus secure much of their nourishment from this source, without resorting to the accumulation of further extraneous nutriment.

However, the problem exists as to how fertilization finally occurs and what means are employed to enable spermatozoa to escape some of the very evident difficulties outlined above.

The spermatozoa. In order to find the seminal receptacle and the contained spermatozoa within the female of Hesperoctenes, that some consideration might be given to these structures, an extended search for them was undertaken. Receptacles could not be found, and the conclusion finally was reached that there were none in this species. The idea of possible parthenogenesis was scarcely tenable, for the males constituted a substantial proportion of my original stock of specimens. The intromittent organ is evidently functional and much resembles that of Cimex.

The spermatozoa finally were found surrounding the tubules near their anterior ends. A recheck revealed them in every female sectioned. They were especially numerous surrounding the germarium and for a little distance below, near the place that earlier had been recognized to be the region in which fertilization theoretically must take place in order to produce the embryonic cleavage stage immediately below.

The numbers of spermatozoa and even their location within the female vary. It is suggested that the youngest females contain more of them than those older. This variation certainly is obvious, but the explanation is not so certain, for generally it is quite impossible to determine the relative ages of several females. My surmise, however, comes from the examination of specimens, one of which can clearly be asserted to be younger than the others. She is youngest because her last nymphal cuticle has not yet been cast aside. In this individual the spermatozoa are found in the places

indicated in figure 2, *s*. These are not the only areas within this female in which spermatozoa are found—simply all that are visible in one section. Other masses occur throughout the abdomen—some, indeed, posterior to those shown, as well as additional clumps in the first abdominal segment.

The nymphal female also showed spermatozoa in great abundance in the paired oviducts and the lower ends of the tubules, in addition to their usual disposition throughout the haemocoel. This was quite a surprise, for in no older specimen could a spermatozoon be discovered there, although this tract was the first submitted to examination. Figure 2 shows this distribution within the oviducts, but only suggests the great numbers that are found there.

Cragg's ('20) careful account of the migration of the sperm cells in Cimex shows that they do not linger within the haemocoel, but pass directly to the spermathecae which they penetrate. Their further movements are always intercellular. The distinction between these hemipteran families Polyctenidae and Cimicidae is that in the former the spermatic clumps are permanently haemocoelous, while they are only temporarily so in the latter.

In all specimens the spermatozoa could be seen lying in the tissues in such a manner that the suspicion arose that they might be found to be embedded in the walls of the reproductive system. This could not be confirmed until the much-thickened wall of the paired oviducts of the youngest female was examined. Here they were found definitely below the surface of the cut tissue. From this it is evident that the spermatozoa can and do penetrate adjacent tissues, perhaps under the influence of certain stimuli. They must, for example, pass through the ovarian tubule to its interior in order to penetrate the ovum.

The spermatozoa are not found in a spermatheca nor does a tissue of any sort surround them. They lie free within the haemocoel of the female insect. At times, and especially in the vicinity of the upper ends of the ovarian tubules, they are found massed between tissue layers in such a manner as to

closely resemble a tubular receptacle with its contained spermatozoa. In Cimex, too, the spermatozoa are not invested with a covering of any sort, according to Cragg.

The arrangement of the individual spermatozoa in these masses seems to be of two sorts. In one they are grouped in a swirl-shaped clump with a hollow core. Sometimes the central portion appears to contain some slightly staining substance, but of its physical or chemical nature nothing can be said. Its apparent presence may be but an artefact due to technique. The spermatozoa in such a mass revolve around the core in a plane at right angles to its axis.

Cragg ('20) describes these clumps of spermatozoa in Cimex as consisting of the gametes directed centrally with the flagella extending outward in all directions, thus giving the mass a somewhat ciliated or flagellated appearance. In this polyctenid the flagellum of each cell continues the curve already projected by the anterior portion, so that the mass would lack this appearance of a flagellate condition.

In the other type the spermatozoa seem to lie at random, often crossing one another, and the microtome knife is equally apt to cut one in section as to cut in a plane parallel to it. Nor are they so densely packed together in this instance as in the former case. In figure 2 they may be seen in one plane as they lie in the oviduct, and at the lower end they are seen massed in another plane at the entrance to the left oviduct which branches off at this point. In both aspects their arrangement is singularly irregular. It would be expected that all would be directed toward the anterior end of the oviduct; therefore they would be arranged with their length directed anteroposteriorly in the lumen.

The spermatozoon is approximately 17 μ in length and very slender, perhaps $\frac{1}{3}$ μ. It appears as a cylindrical thread-like cell with a slightly tapering anterior end. The posterior portion tapers very gradually into the flagellum. No details could be seen and even the exact measurements are wanting, for it was impossible to determine whether any particular gamete measured was entire. Many, however, proved to be

of the length recorded. That they were actively motile at the time of fixation is indicated by their curvature when viewed under the microscope.

Conclusion. From the foregoing we may safely surmise that the problem of fertilization is not solved in the customary manner of the insects as a whole, but evidently a specialized mechanical process is involved. Later it will be shown that some of the nutrient organs, first the serosa and later the pleuropodial extensions of the embryo, are closely adherent to the walls of the ovarian tubules and oviducts. It would be difficult to explain how spermatozoa might mechanically pass two or three embryos with such attachments. The physiological consideration of their immunity to absorption would also add to our difficulties in such an explanation. There is at present no optical evidence that spermatozoa ever are found in the lower ends of the tubules or the oviducts in the mature female. In fact, the descent of the embryo would be expected rather to sweep them down the reproductive tract to the exterior.

The possibilities seem to be these:

1. The introduction of spermatozoa and their migration up the reproductive ducts might occur in the usual manner.

2. The introduction of spermatozoa by impregnation and their migration may take place as usual in most insects, but upon reaching the paired oviducts the male gametes may penetrate their walls and escape into the haemocoel. The female may mate but once, or she may mate frequently as in Cimex and many other Hemiptera. The spermatozoa may gather in the haemocoel, where they are kept alive during the entire reproductive period of the insect.

3. An organ similar in function to the organ of Berlese might be present and serve to introduce the spermatozoa into the haemocoel.

The objections to the first type of fertilization process have been presented. Further researches under laboratory control subject to verification by freshly fixed specimens must be made before this possibility could be sustained. There might

very well be an opportunity for the spermatozoa to pass an embryo in the oviduct when the cuticular layer is secreted about the pleuropodia. It might also be possible for them to pass younger embryos at intervals, as, for example, when the embryo moves down in the tubule into the oviduct, when revolution of the embryo occurs, and thirdly, before the serosa is formed. Ascent at these times would permit the spermatozoa to attain the upper portion of the tubules.

In the second suggestion we find nothing in conflict with cases already known in the genus Cimex. Here the sperm cells, having entered the haemocoel through the organ of Berlese, penetrate the walls of the tubule to reach the ova. In my own observations I seem to find these gametes embedded in the walls of the reproductive tissues. This case, then, simply assumes that they pass into the haemocoel in a manner quite analogous to that found in the bedbug, although in the latter they continue their migration in the tissue instead of returning to the haemocoel.

The assumption that the female may mate but once is plausible, for it fits with the observation already given that the young female seems to have the most spermatozoa, while those older vary in the number. As might be expected, too, the clumps are distributed throughout the entire haemocoel of the young female. In the older insects they are found grouped more closely about the tubules near the anterior part of the abdomen. Some have very few spermatozoa remaining. Whether this is due to lack of opportunity to secure fresh supplies from males, whether the female is reaching the termination of her reproductive period, or simply that the original and only possible supply of spermatozoa is greatly diminished are questions yet to be answered. Any one of these contingencies might account for the fact.

We should also take into account the rarity of this insect. It is possible that in the majority of cases at least, after leaving its original host for another, the female may not often inhabit a bat containing a male insect. It may be that the original and only impregnation with spermatozoa is

secured from the male offspring who are born of the same mother and only at rare intervals does outbreeding take place.

The last alternative offered has much to commend it to our consideration. If the organ of Berlese had not been discovered in Cimex, a genus in a related family, the idea would not have been suggested. While the evidence of the spermatozoan behavior in Hesperoctenes accords in part with that of Cimex, one difficulty remains. No organ to correspond in function to the organ of Berlese has been seen. Jordan ('22) remarks that his search for Berlese's organ in Polyctenidae yielded negative results and "there is nothing in the female abdomen of the Polyctenidae to indicate that copulation does not take place in the ordinary way of Rhynchota." While I, too, have failed to locate such an organ, it is still possible that some analogous structure may eventually be found.

This leaves the question still open as to just how the spermatozoa are received and their history within the female is but partially revealed. Of the possibilities offered by the writer, the evidence is equally favorable to any one of the three at the present time.

Paedogenesis. All of the offspring are born during the physical maturity of the mother. This statement, however, does not express the true conditions concerning ovulation, fertilization, and the early development of some of the offspring to be produced. Uichanco ('24) was confronted with precisely the same phenomenon in Aphididae. He was able to show that in parthenogenetic females the reproductive organs release mature ova which commence their development before the physical maturity of the individual is reached. Giard's views on the complete independence in development of physical maturity and the reproductive function are quoted by him. He contends that while the offspring are born of physically mature individuals, yet their discharge from the germaria as mature ova took place prior to this period. Such young, therefore, should be considered as truly paedogenetic.

An examination of figure 2 reveals a somewhat diagrammatic sketch of a young female. It is evidently a nymph, for the loosened exoskeleton still surrounds it almost intact. The hypoderm has secreted a new cuticular layer beneath it, which has been represented in black to distinguish it from the older cuticle. The two differ in staining reaction, showing that there exists some chemical difference between them.

The new cuticula presumably is the adult investment in which other substances have not yet been deposited. Based upon previously accepted criteria, this would establish the approach, but not the attainment of physical maturity. Internally, a glance shows clumps of spermatozoa in various parts of the body. Hence some form of mating must have occurred in the physical immaturity of the animal, for the species is dioecious.

Scrutiny of the reproductive organs reveals that ova have matured and have been released for some time. Fertilization has occurred in several of them and the earliest embryonic stages are visible. Doctor Wheeler called my attention to this condition in an early examination of the slide and pointed out its possible significance.

Careful check of the ovarian tubules gives the following ontogenetic stages present in the reproductive system: three oocytes, very small in size, one oocyte (secondary oocyte stage?), one egg, second cleavage stage, one egg, blastoderm formation, and one embryo in germ-band stage prior to the formation of embryonic envelopes.

This list shows that the first few individuals to be extruded by the mother must be considered as paedogenetic in development. The number may vary in different individuals, though there are at present no other specimens available for comparison.

Uichanco proposed a classification of the known types of paedogenesis in which Hesperoctenes, too, may be placed with the addition of a single subdivision. His arrangement includes in the second class with unisexual paedogenesis all of the paedogenetic insects heretofore listed. Manifestly,

Hesperoctenes does not belong here, being bisexual. In his first class, however, those listed produce only fertilized ova. Hesperoctenes is, on the contrary, a bisexual viviparous paedogenetic species. To complete his excellent classification by the addition of this insect in its proper position, it is suggested that his class 1 be slightly changed in wording to avoid its present unnecessary limitation to oviparous forms. It would be necessary to divide it into two subclasses, 1a and 1b (of which 1b is new), to care for the examples to be cited. It appears then in abbreviated form, and disregarding the word derivations, as follows:

I. Paedogenesis. "Definition: That method of sexual reproduction whereby during the immature stages of the mother the ovum reaches a condition which enables it to begin a new germ-cell cycle."

1. "Bisexual paedogenesis. Definition: That form of paedogenesis wherein the union of biparental elements accompanies reproduction."

a. Bisexual oviparous paedogenesis. Definition: That form of bisexual paedogenesis in which the preadult individuals "attain sexual maturity and produce eggs and sperms. Examples: Bolina hydatina (Ctenophora) and Ambystoma tigrinum (Chordata) (Shull. 1920, pp. 181–183)."

b. Bisexual viviparous paedogenesis. Definition: That form of bisexual paedogenesis in which the development of the ovum takes place in utero during an immature stage of the mother but the offspring are produced in a stage of development subsequent to the egg. The mother meanwhile has attained sexual maturity. Example: Hesperoctenes fumarius (Hexapoda).

2. "Unisexual paedogenesis. Definition: That form of paedogenesis wherein the development of the ovum takes place without fertilization. As I have pointed out in the foregoing discussion, in the viviparous forms, the extrusion of the ovum from the germarium and the subsequent intrauterine development thereof correspond in their biological significance to oviposition and the subsequent incubation of the eggs in the ovipara."

a. "Unisexual ectopaedogenesis. Definition: That form of unisexual paedogenesis wherein the externally visible manifestation of the end products, in the form of extraovarian extrusion of eggs or young, occurs during the preadult stages of the mother. Examples: Certain groups of Itonididae, like Cecidomyia (in some of the species only) and Miastor."

b. "Unisexual entopaedogenesis. Definition: That form of unisexual paedogenesis wherein the development of the ovum takes place in utero during the immature stages of the mother, and consequently the externally visible manifestation of the end products, in the form of extraovarian extrusion of eggs or young, does not occur until subsequently. Examples: Parthenogenetic Aphididae."

There is no evidence to present regarding the state of maturity of the male in these matings or whether sexual maturity only follows the physical maturity of the soma. It would be of interest and of some value if we knew whether this pushing forward of the reproductive function occurred in the female alone or whether a functional precocity had appeared concomitantly in the other sex.

Embryogeny

The ovum. The source of the ova is not definitely determined. It is suspected that they arise from the region of the transversely placed cells at the apex of the germarium, as these nuclei are slightly larger than those forming the epithelial layer lower in the dilated portion. Further, a careful scrutiny of the walls of the germarium failed to reveal any nuclei that showed characteristics not possessed by all of the other nuclei of this region and none were surrounded by excess food material. Finally, the youngest ova invariably are found in the apex of the germarium as it narrows to form a column of cells penetrating the core of the terminal filament at the latter's junction with the ovarian tubule.

The young ovum is oval, with more or less acutely tapering ends. It lies in the lumen of the germarium and evidently here receives its first supply of food material. Many were found in this place in various germaria, but all had some accumulation of nutriment. The smallest ovum discovered measured 14 μ in length and 6 μ wide. It was pyriform in shape, with its tapering end anteriorly directed at the apex of the germarium (figs. 11 and 12). Only one is formed at a time and it moves down into the vitellarium before a second is released.

The young ovum as it passes down the vitellarium is ovoid in outline, with a large oval subcentrally placed nucleus (fig. 13). The cytoplasm appears to be highly vacuolated, which is doubtless due to inclusions of finely divided fat-droplets. The vacuoles, being highly refractive, cause the cytoplasm to appear reticular. The cytoplasm takes the stain quite like the epithelial layer of the ovariole, but both stain deeper in color than the maternal muscular tissue, digestive epithelium, or cells of the fat-body. The cytoplasm appears denser and less alveolar immediately surrounding the nucleus. The lighter color and the greater number of peripheral vacuoles may be due simply to the lesser thickness of the egg near its margins, but in the microscope it certainly does not give the appearance of being a perfectly homogeneous mass. The greater width of this ovum is 24.5 μ and the lesser measures 17.5 μ.

The nucleus is conspicuous and apparently quite clear. Its length is about two-fifths that of the greatest dimension of the egg. A mass of densely stained chromatic material occupies a large subcentral portion of it, indicating that it is going through the maturation stages at this time. No other chromatic substances are visible in the nucleus with my stains, and the clear nucleoplasm fills the rest of the nucleus.

The egg described above is found in the upper end of the ovarian tubule whose lumen is but slightly greater in diameter than the former. It is interesting to note that the cytoplasm of the epithelial layer lining the tubule appears to be structurally identical with that of the ovum, except that there is no marked increase in density of the cytoplasm adjacent to the epithelial nuclei. The epithelial cytoplasm as a whole is stained very slightly lighter in color. The nuclei, too, are not quite so large, though they are enormous compared with most other maternal nuclei. They have the chromatin so distributed that the separate granules or masses of chromatic material are distinctly visible. The proportionate size of the epithelial cells should here be noted in comparison with the conditions nearer the germarium. The epithelial layer

at this point is much thicker than in the lower part of the vitellarium, the approximate thickness being 9.8 μ. The greater diameter of the tubule is 58 μ, while the lesser width is 40 μ. This fact emphasizes the appearance of the nuclei which occupy about half of the epithelial space.

When the ovum is fully mature, it measures 125 μ in length and 48 μ in width. It entirely fills and even somewhat dilates the tubule in which it lies (fig. 14). The cytoplasm is finely granular, the contained nutriment causing it to appear lace-like because of the refractive, clear droplets within its meshes. The vacuoles are oblong in shape, their greatest dimension paralleling the greatest dimension of the ovum. There is no follicle surrounding the egg. For this reason no chorion or egg membrane is present—a condition resembling that found in Hemimerus. The germinal vesicle is very large and is situated near the anterior end of the egg. It measures 25 μ in length and 16 μ in width. The numerous chromatin flakes stain deeply. All are peculiar in being spherical in shape, but they vary greatly in size. No linin could be seen connecting them with one another. The delicate nuclear membrane is very distinct when properly illuminated, otherwise it is practically invisible. The ovarian tubule is contracted both before and behind the egg. The epithelium consists of a dense layer of cells which now are thinner than those shown in figure 13, but the nuclei appear to be the same. No cell walls could be distinguished.

Maturation of the ovum. No cytological study of the maturation stages could be made because of lack of material, including male specimens for spermatogenesis. Also, the absence of proper fixation in the original preservation of the material precluded reliable observations being made.

It was recognized that maturation stages commenced immediately upon the release of the ovum into the germarium. All of the early ova in my sections show that phenomenon. The chromosomes never appear as discrete rods, but always are massed in irregular clumps of which no details were visible (figs. 6, 11, and 12). When the ovum is mature with

its reserve of nutriment as shown in figure 14, the nucleus is in the resting stage.

One conclusion may be drawn from the above. The descent of the ovum into the vitellarium is rather rapid or the ovarian nucleus would be found at times in the resting stage in the germarium or the upper end of the vitellarium.

Cleavage and formation of the blastoderm. The zygotic nucleus undergoes cleavage presumably within the immediate vicinity of the anterior end of the egg (fig. 15). This is also the case in the aphid, Macrosiphum rosae, as Uichanco ('24) recently has shown. This assumption of the anterior location of the nucleus is made because it would be unlikely that the male gamete migrates the entire length of the ovum or would pass around it to the posterior end before entering. There being no chorion over the ovum, entrance may be made at once. The two mature ova discovered contained the female pronucleus in the anterior end of the egg and the nurse cell mass followed behind the egg. Successive mitoses result in the formation of a number of cleavage cells, some of which remain in the interior, while the others migrate to the periphery of the egg. This is shown clearly in figures 16, 17, and 18. While spireme stages apparently were distinguished at the periphery, other phases of division were not discernible and it is not certain that the superficial nuclei continue to divide. Mitoses, showing different stages, were found within the central portion of the egg. While most of the superficial cleavage nuclei were in the resting stage, all were in process of mitosis in the interior.

The cytoplasm of the egg may well be considered at this point. While appearing in the unfertilized ovum as an alveolar body with rather evenly dispersed fat-droplets embedded in a matrix of cytoplasmic material, this is not the appearance during cleavage. The cytoplasm and its fatty inclusion have undergone a comprehensive realignment. Most of the former has concentrated in a thick sheet or layer at the surface of the egg, while the nutrient material is centrally situated. This peripheral cytoplasm, termed 'Keim-

hautblastem' by Weismann, 'periplasm' or 'perivitellus' by Korschelt and Heider ('99), is remarkably thick, which is not the usual condition in insects, as pointed out by the latter authors. The cleavage cells first reach the periplasm at the anterior end of the egg and here embed themselves within this dense layer of cytoplasmic material. Rather large, more or less cuboidal cells are promptly constricted off, so that the periplasm eventually becomes cellular in nature. Cellular differentiation is not incomplete as indicated for most insects in this stage, but each blastomere is completely separated from adjacent cells and at first quite an interval often intervenes between them (figs. 16 and 17). This condition is quite similar to that in aphids, according to Uichanco ('24). Unfortunately, Heymons lacked these stages in his study of Hemimerus, so that complete data of blastoderm formation are unavailable in this unique insect. He believes, however, that cleavage is incomplete, as in Forficulidae in general. This is quite a contrast with Hesperoctenes fumarius, especially as both insects are alike in being yolk-free. Complete cleavage is rather to be expected. Heymons' ('12) drawings (Taf. 8, Fig. 3), however, show that in the Hemimerus egg there is no distinct periplasm developed and the cytoplasm possesses a uniform frothy or foamy appearance throughout.

The cytoplasm left behind during the formation of the periplasm and the blastomeres is quite scanty. It at once accumulates around the remaining cleavage nuclei and only attenuated strands connect the cytoplasmic 'islands' with each other. The nutrient material of the egg fills the interior.

The question of the origin of the vitellophags often has been discussed. Some writers have claimed that they arose from the interior cleavage nuclei, while others maintain that all nuclei migrate to a superficial position and it is only later that a few return to the interior. This procedure recently has been very adequately discussed by Uichanco in relation to the aphids. I agree with him that the subject apparently is of doubtful morphological importance; nevertheless, it is regretted that I could not trace the origin of the vitellophags

or trophocytes from the first cleavage to later stages. The whole matter appears of interest simply from its historical significance. My observations covering quite early cleavage stages show at least that from this time on vitellophags (trophocytes) or cleavage nuclei are present continuously within the interior cytoplasm of the developing egg. The term vitellophag is used to distinguish cells that are active in elaborating nutrient material from the yolk contained within the eggs of most insects. Inasmuch as the egg of Hesperoctenes fumarius is practically yolk-free, it is considered more appropriate to use the term trophocyte to indicate these cells in this insect. I have accordingly done so throughout the remainder of this paper. In adopting this term we are following the suggestion of Heymons ('12) in his work on Hemimerus.

None of the stages shown in my material gives a clue as to the conditions surrounding the embryo upon completion of blastoderm formation. The posterior pole in parthenogenetic aphid eggs has no blastoderm, and to what extent this is duplicated in Hesperoctenes fumarius is not at present known. In the latter species blastoderm formation has been seen extending posteriorly to about the last half of the egg. Mitotic divisions are visible near the posterior pole and the blastoderm stage evidently was continuing at the time of fixation of the specimen.

In the posterior portion of the egg during cleavage are found two very large nuclei, quite vesicular, with prominent chromomeres and linin. These can only be accounted for by suggesting that they might be the primordial germ cells of the embryo. They are so transparent that slight overstaining or understaining may readily obscure them. No other nuclei discovered in this insect are like them except the pronucleus of the ovum. They are not quite so large as the latter, linin threads can be seen, and the chromomeres are irregular flakes. These differences might easily be explained in view of the future before them. The pronucleus is preparing for the process of fertilization, while these cells are to

be more or less quiescent until the maturity of the individual occurs.

Segmentation and the appearance of the extremities. The germ band soon becomes long and rather flat, though multilaminar, due to the superimposed condition of the ectodermal cells. The width of the cephalic portion is about twice the anterior abdominal width. The enormous lateral expansion of the optic lobes, so characteristic of most insect embryos, is absent here. There is apparently but little increase in the thoracic width over the preceding stage. The embryo assumes a characteristic 'S' shape, with the cephalic lobes flexed sharply dorsally, so that this region lies directly in contact with the mandibular region. The terminal abdominal third of the body, on the contrary, is flexed ventrally, so that the posterior half of the abdomen comes to lie opposite the ventral ectodermal surface of the anterior half of it. The flexure of different embryos varied in details, perhaps because of the space available to each embryo and in part perhaps because of the slightly different ages of the embryos viewed in this stage (figs. 20, 21).

During this development of the germ band typical segmentation furrows have appeared as transverse ectodermal invaginations. This comes about either as the result of the rearrangement of the nuclei and cytoplasm in definite places, so that the germ band is thinner there, or else active proliferation of cells has caused swellings to appear at intervals, corresponding to the future segments. The definitive mesodermal layer seems not to be involved in early segmentation at least. As usual with insects, the appearance of segmentation posteriorly lags behind the regions cephalad. Also the posterior constrictions are not so sharply defined as the anterior invaginations.

As segmentation occurs the stomodeal and proctodeal invaginations ordinarily arise. In the sections available the former could not be found, but the latter was detected readily (fig. 22). The proctodeum is at first a rather large opening which later is much reduced in diameter.

The appendages arise as paired ectodermal thickenings. At first the mandibular, maxillary, and labial swellings are equal in size to the thoracic evaginations. The pleuropodial rudiments are indistinguishable from the thoracic evaginations, but the succeeding anlagen are comparatively insignificant, appearing more as segmental swellings. The prothoracic appendages are always in advance of the others in development. After developing the proximal portions perpendicular to the germ band the distal parts are deflected posteriorly. They are followed in turn by the other thoracic appendages. The pleuropodia evaginate until they almost come in contact with the amnion (figs. 20, 25). This seems to be the limit of their present development until after their invagination within the formative haemocoel of the embryo.

Early in this stage the mesoderm arises from the ectoderm. Just how this process takes place could not be determined, but figure 20 shows a mesodermal sheet dorsal to the embryonic rudiment. From its appearance one might conclude that it arose simply by delamination from the ectodermal cell mass.

The mesodermal tissue invades the ectodermal evaginations very early, so from the first the appendages possess mesoderm. At the beginning the mesoblast cells are irregularly arranged. This condition speedily gives way to a more definite organization in this tissue, as shown in figures 23 and 24, where the mesoblast cells may be seen as an inner mass of cellular material.

Prerevolution stage. Just before the revolution of the embryo, the embryonic membranes are intact except that the amnion is not visible directly opposite the pleuropodia, as shown in figure 30. The embryo has straightened out somewhat, so that the characteristic shape of the germ-band stage is almost lost, though the last segments of the abdomen still are flexed. The extreme cephalic region also has a dorsal fold. The proctodeal invagination readily is found, but I am still unable to find traces of the stomodeum. The limbs project posteriorly, but reach only to the first four abdominal segments. The posterior abdominal region is flattened and

lacks the roundness that is becoming evident in the thoracic and anterior abdominal segments. This assumption of a more cylindrical shape comes about through the growth of the lateral portions of the embryo.

The ectoderm ventral to the nerve cord is definitely one-layered, but laterally it is irregularly so. The ectoderm of the limbs is arranged apparently in two layers, but this is quite indefinite, too. No evidence of tracheal buds could be seen.

The mesoblast cells found in the extremities are segregated into one to three groups of cells. These extend to the tips of the appendages, but fuse into one common mass before terminating there. They are not attached to ectodermal cells at their distal ends, but lie free in the lumen of the appendages. Neither are they definitely arranged at this time to form the muscle fibers. Such specialization appears shortly after revolution has taken place.

A few mesoblast cells lie scattered in the region of the future haemocoel. There are none in the pleuropodia, as these organs now are of the invaginate type.

The trophocytes are prominent, but not so numerous as the smaller mesoblast cells. They are found dorsally, where they seem to form intimate contact both with the serosa and the embryo. This is shown in figure 24, which is an embryo in an early stage of development with evaginate pleuropodia, and in figure 30, which represents a very late embryo in the prerevolution stage with the pleuropodia invaginated.

In the region of the first abdominal segment of a late embryo in this stage the invaginated portions of the pleuropodia fill almost the entire space and extend into the thorax as well as into some of the abdominal segments (fig. 30). Their median margins are in contact with one another.

The nervous system consists of a very large mass of cells extending the length of the embryo. Posteriorly, however, they are not so conspicuous as in the cephalic and thoracic parts of the body. In the anterior regions of the embryo a bilobed fibrous area has appeared centrally in this cell mass

which marks the early formation of longitudinal nerves. This inner portion of the nerve cord is comparatively quite restricted in diameter and extends through the cephalic and thoracic regions. Longitudinal nerves are not yet formed in the abdomen.

Blastokinesis. About the time that the prothoracic appendages have developed almost half of the body length, the embryo becomes considerably shorter and broader. This shortening arises in part, at least, through the coalescence of some of the body segments, particularly those comprising the cephalic region. The width comes about in part through the lateral growth of the body walls. At this time the embryo undergoes reversal, so that when it again comes to assume a position in which its length lies parallel to that of the mother, its cephalic region is directed forward in the maternal body. As a rule, it will be found lying on its side. Only at the very latest stage does it again shift, so that it finally lies dorsal side up at birth.

Revolution seems to occur at a point where the tubule runs transversely in the abdomen of the adult or else the reversal of the embryo's position makes the tubule bend transversely at this time. It may be that this is the junction of the ovarian tubule and the oviduct. It is peculiar that, irrespective of the crowded condition, resulting even in considerable distention of the abdominal region of the mother, the embryo seems to have ample room for this process. It is interesting to point out the comparison between Hesperoctenes and Hemimerus at this time. The latter is shown near the completion of blastokinesis in Heymons' paper ('12), figure 3. The amnion and serosa are not destroyed during this process, for the embryo is employing them as nutrient organs. The dorsal body wall is closed except at the cephalic vesicle. The enteron is formed and connects with both stomodeum and proctodeum. In Hesperoctenes these events occur later in the embryonic history, and in addition the serosa and the amnion are ruptured and cease to function.

Postrevolution stage. This stage in embryonic development is characterized by the ruptured embryonic membranes, which are located anteriorly, and the dorsal growth of the body walls and their fusion along the dorsal midline. They are not fused in the cephalic region at this time nor is there any sign of the mesenteron. Figure 34 shows this in a longitudinal section near the midline of such an embryo. The metathoracic limbs extend to the seventh abdominal segment and the total length of the embryo is 475 μ. The nervous system consists of paired, segmentally arranged ganglia that are united by the broad, paired, longitudinal nerve trunk. Externally, these appear to be fused into a single cord. The following enlargements due to ganglionic material may be found: supra-oesophageal ganglion, suboesophageal ganglion, three thoracic, and there appear to be ten abdominal ganglia.

The ectodermal cell walls are not visible. The cells apparently are small and appear to be cubical in shape. The nuclei are very small, measuring about 1.3 μ in diameter (fig. 29). The ectoderm is one-layered, but the stomodeal and proctodeal invaginations are irregularly so. At the inner end of the latter the malpighian tubules have formed and appear to be four in number. Aggregations of cells at the inner extremities of the stomodeal and proctodeal invaginations suggest the presence of formative endodermal cells. Similar cells scattered through the formative haemocoel presage the beginning of the definitive mesenteron. Their multiplication and final disposition to form the midintestine have not been followed.

A few mesoderm cells are present in the haemocoelar space, but seem not to have increased at the same rate as the ectodermal tissues. They lie in a loose sheet dorsal to the central nerve chain. I can see no evidence of their forming segmental masses of fat-body cells at present. Some of the limbs show the arrangement of mesoderm cells into sheets or formative muscle fiber strands, but no evidence of the definitive muscle fibers has yet appeared. A section of

one of these formative strands from an embryo just after revolution is shown in figure 41. The cytoplasmic strand is barely as great in diameter as the ovate nuclei embedded in it at frequent intervals. No cell walls can be seen and the nuclei are placed with their long axes parallel with the strand. Their chromomeres are gathered at the poles.

The long and constricted haemocoel is largely filled with the inner ends of the pleuropodia. The remaining space is occupied by a few very large trophocytes and many smaller cells whose nuclei typically contain three prominent nucleoli. The latter may really be formative fat-body cells because of the resemblance of their nuclei to the fat-body nuclei, but this could not be determined.

The free embryo. Under this heading we may consider the most unusual situation of the embryo brought about by its peculiar embryological history. It will be recalled that the ovum had no egg follicle cells surrounding it, and in consequence no chorion or eggshell was secreted. The egg was naked. The developing embryo soon was enclosed by the serosa and partly covered by the amnion, both of these membranes originating from the egg itself in the process of embryonic development. Only these embryonic membranes separate the embryo from the walls of the ovarian tubule. During blastokinesis these membranes are ruptured and removed to a position anterior to the embryo. Consequently, the latter now lies free in the oviduct without external covering and directly in contact with the walls of the oviduct.

The pleuropodia, of course, are growing outward and around the embryo at this time and soon enclose the latter in an envelope quite as effective as a protecting cover.

During the interval between the rupture of the membranes and the extension of the pleuropodia around the body there are no known means available for the embryo to secure nutriment from the parent. No other case similar to this can be recalled in insect embryology. Even Hemimerus has egg follicle cells, although they secrete no chorion. These cells serve as a placental organ in the nourishment of the young

and continually surround the embryo, where they uninterruptedly act in lieu of the eggshell as a protective envelope.

Postrevolution stage, half-grown embryo. This stage follows the postrevolution stage that has already been described. The embryo has grown to a length of about 600 μ, as shown in figure 36. The stage is characterized by the formation of the mesenteron, which by its fusion with the stomodeum and the proctodeum has brought about the completion of the digestive tract. A short account of the cells forming the mesenteron in embryos intermediate between this and the preceding stage and leading to the condition found in the half-grown embryo follows.

The junction of the mesenteron with the intestine is interesting, as it shows that the former projects into the latter for a short distance at this point. Also the malpighian tubules, which are proctodeal in origin, connect with the intestine just at the point of fusion of the latter with the mesenteron. The newly formed mesenteron is shown in figure 37. The cells are subquadrate, with faintly staining cytoplasm which is highly vacuolar. The cell boundaries are visible, probably through shrinkage. The diameter of the individual cell is about 4 μ. The nuclei are small and stain deeply, showing no details. Ectally, the mesenteron is bounded by a thin sheet of cells without distinct boundaries. The nuclei are very small and stain black. These later are to form the thin single layer that surrounds the midintestine in mature specimens.

A section of the mesenteron from a slightly older embryo of the same stage, perhaps not quite half-grown, is shown in figure 38. The epithelial cells have become much flattened, and each contains one or two very large vacuoles that completely fill the interior and force the cytoplasm to a marginal position. There is no sign of a musculature surrounding the digestive epithelium and the basement membrane is not visible.

When the embryo is about half-grown or a little older, the enteric cells appear as in figure 39. They are assuming the columnar shape of the adult. Each cell is highly vacuolar and

the nucleus, ovate in shape, is situated at its base. A delicate basement membrane was present, and a very flat epithelium with minute, darkly staining nuclei appeared to invest the mesenteron externally. No muscular layers could be distinguished.

Within the anterior third of the mesenteron, the embryonic membranes are found densely compacted into a cylindrical mass with bluntly rounded ends. The nuclei of these membranes are especially numerous in the peripheral region, while only cytoplasm occupies the central portion.

The trophocytes are enclosed also and are found posterior to the serosa and amnion. The reason for this is obvious. As the trophocytes elaborate nutrient substances from these membranes the material passes down the alimentary tract and is absorbed in the posterior portion of the mesenteron.

The nervous system has been concentrated anteriorly until only four pairs of very large ganglia appear. These are united by broad bands of the oesophageal commissures and the longitudinal nerve cord. The origin of each ganglion could not be followed, but the supra-oesophageal ganglion seems to be composed of three parts, the two smaller anterior lobes probably being concerned with the special senses in the antennal and oral regions. Apparently the suboesophageal and first thoracic ganglia are fused into the second large ganglion, while the mesothoracic and metathoracic ganglia form the anterior half of the remaining ganglionic mass. The posterior half of this last ganglion is only slightly marked off from its anterior portion and consists of the abdominal ganglia. The nervous system still exhibits its paired condition throughout, but superficially this cannot be seen.

The fat-body is distinctly segmentally arranged. The cells are highly vacuolar, each containing only three or four large vacuoles. The nuclei appear to contain three large nucleoli, as shown in figure 46, but I cannot distinguish linin or chromomeres in them. Along the dorsal abdominal region a few enormous oenocytes are arranged. Each stains a bright pink color with eosin. The cytoplasm exhibits no physical structures. The nuclei are small.

The limbs reach to the tip of the abdomen and the general segmentation of each is indicated by definite constrictions. No tracheal invaginations could be found in the abdomen.

The main thoracic musculature is slightly in advance of the other parts of the embryo. The nuclei now are distributed over the surface of a relatively large fiber (fig. 42). The nuclei are still ovate, but flattened. The chromomeres are few in number, stain strongly, and are scattered.

In the appendages the disposition of the mesodermal cells is not so regular. The myoblast cells of the preceding stage have arranged themselves end to end, indicating the formation of the future fibers. Their extremities adhere to ectodermal portions of the embryo.

Precuticular stage. This embryo is quite like the succeeding stage to be described, but it is desired to record the advent of new structures that have made their appearance at some time prior to this. While there is no cuticula present, the embryo is about the length of the oldest embryo at the time it is ready for birth, namely, 1.3 mm. The extremities have grown posteriorly to their length at birth, the mesothoracic limbs cross the ventral surface of the abdomen to the opposite side. The metathoracic limbs even curve back and then extend forward to the fifth abdominal segment.

The digestive system contains no trace of the embryonic membranes, and the trophocytes, too, are digested and almost entirely absorbed.

The musculature is complete, yet the segmental muscles of the abdomen are not fully mature. The thoracic muscles as well as those of the limbs appear almost as mature as the muscles of the nymph. Figure 43 reveals a portion of a large fiber with the nuclei strung along the center in a linear arrangement. The nuclei are round and the few chromomeres or nucleoli stain very deeply. Striations are apparent, but the core occupied by the nuclei was not striated. No further details could be seen. Ventrally located oenocytes seem to be present in numbers in the region of the epineural sinus.

The tracheal system consists of spiracular openings segmentally arranged connecting to the main longitudinal trunk. The cells composing the trachea are arranged in a single layer. They are long and very flattened, with much-flattened nuclei. I could find no tracheoles.

The first evidence of the gonads is found in the late stages of the full-grown embryo, but before chitinization has taken place. These organs are paired, tubular structures of cylindrical shape.

Their outer covering is a one-layered epithelium which extends forward to form the tubules. This tubular extension of the gonad seems to be paired in the specimens studied.

Within the gonadal epithelium are found the germ cells. These are cubical in shape, with a finely granular cytoplasm. The nuclei are very large, exceeded only by the nuclei of the trophocytes. The appearance of the nucleus is characteristic and is shown in figure 48.

The gonads are located near the middorsal region of the abdomen directly above the anterior third of the mesenteron.

The cuticular stage. This stage is characterized by a degree of development that corresponds very closely to that of the newly born nymph. The cuticle is secreted in a very definite layer over the hypoderm, but none could be seen in the digestive tract. Presumably the cuticle was present, but not distinguishable. The embryo has assumed the final position for birth, with its axes corresponding to those of the parent. Surrounding it is the cuticular secretion from the pleuropodia. This envelope serves as protection to the maternal reproductive tract and perhaps tends to assist in eclosion by covering the setae of the offspring.

The cells of the mesenteron are tall columnar in shape and densely packed together. The digestive tract is coiled only in the intestinal region. The malpighian tubules are now very long. They have from the first been tubular with large cells containing prominent nuclei. In cross-section it is seen that two or three cells of the tubule form a ring enclosing the lumen of the organ.

The cells of the tracheal system are exceedingly thin. The chitinous intima is only visible because of the reflection of light from the taenidia.

The muscular system still remains immature in the intrasegmental fibers, but elsewhere is fully developed. As it is so similar to the muscle fiber of the mature insect, one of the thoracic fibers may be described here which will serve to illustrate the condition in the adult as well as in the embryo ready for birth.

A cross-section of three muscle fibers in the limb of a chitinized embryo is shown in figure 45, while a longitudinal section of the adult muscle fiber in the thorax is represented in figure 44. Here the striated appearance shows as broad transverse bands with lighter-staining intervals between. The nuclei are large, subcylindrical, and greatly elongated. The chromatic material is concentrated at the two poles of each nucleus. Further details were not visible.

The development of the muscle fibers of the limbs seems to follow the same course that has been described for the thoracic muscles in Hesperoctenes. This condition is interesting, in view of the statement by Imms ('25, p. 49) wherein he says that the leg muscles of insects have their nuclei peripherally located All voluntary muscles of Hesperoctenes appear to have their nuclei in a central position.

The reproductive system is practically complete, but not mature in its appearance. The paired oviducts are directed ventrally and posteriorly, as in the preceding stage, but their fusion with the common oviduct has not taken place. The condition that exists just prior to fusion is shown in figure 47. Whether fusion takes place before birth or later is not known. The gonads still remain two in number. Each contains about fifty germ cells.

Orientation of the embryo. Just before birth the nymph is found to be so oriented that its axes correspond to those of the parent, that is, the ventral surface is ventral in relation to the mother's body. The longitudinal axis of the young at this stage actually only approximates that of the mother.

While the head of the offspring projects anteriorly and may be seen to one side of the midline of the parent, its posterior end is directly opposite the genital opening; thus its antero-posterior axis slightly diverges from that of the mother. The reason for this appears to be due largely to the presence of another embryo or younger nymph in the anterior portion of the maternal abdomen which tends to press the first individual to the opposite side. The oldest nymph extends from the middle of the parent's first abdominal segment to the anterior part of the last visible segment. The width of its body is about half the greatest abdominal width of the parent. This appears in figure 49, which is a view of a whole mount of the abdomen of a gravid female. No stains have been used.

At this time certain interesting details of the offspring's position are visible to the observer. Its head is flexed ventrally beneath the large prothorax; its long slender legs are directed anad to the last segments of its abdomen, where they bend and extend across the posterior abdominal segments. Thus the tarsi and portions of the tibia of the legs from the right and left sides overlap one another in the caudal regions. The outlines of the pleuropodia and their extensions, too, are visible, as shown in figure 50. Segmentation, too, can clearly be distinguished in the embryo.

The nymph now is in position for birth. The cross-section of its body most nearly agrees with the genital opening in so far as the greatest width of the nymph corresponds to the greatest dimension of the transverse slit of the vaginal opening. Its extreme width, however, is still very much greater than the apparent size of the opening through which it must pass out into the world, so that, compared with oviparous forms, every birth in this species is a real achievement. The tip of the abdomen is the first part to appear, while the head is the last to be extruded. Heymons ('12) already has described this birth position as being typical for insects. His statements are especially pertinent here, as he is treating the manner of eclosion in Hemimerus, which is viviparous and whose offspring is relatively very large, corresponding in

these particulars with Polyctenidae. Actual extrusion of the young never has been recorded for Hesperoctenes or for Hemimerus. Judging from the size of the embryos in this study, it may safely be concluded that it would be impossible for the embryo to reverse its position. It is longer than the width of the mother's abdomen, another large embryo is wedged in beside its anterior half, and, finally, there are morphological details of embryonic envelopes to be described later which would interfere with marked changes in position.

The second oldest embryo will be about half-grown when the oldest appears as described above. It extends from near the cephalic margin of the metathorax of the parent posteriorly to the caudal margin of the third abdominal segment. The dorsal side is pressed against the older embryo, while its ventral surface faces the lateral margin of the mother's body. The limbs seem to extend only to the tip of the abdomen. Due to the pressure of the older nymph, its anteroposterior axis also does not quite correspond to that of the mother, but it is parallel to its sib. However, the abdominal portion of the younger lies nearest the pleural region of the mother, while the cephalic portion of the oldest one approaches the side of the mother's abdomen.

The relative positions assumed by the embryos of successive ages in regard to one another seem to be entirely a matter of chance. Both whole mounts and cross-sections of individuals containing two to four embryos showed the latter to be different in orientation every time. Some were found that were developing apparently on their sides, others were upside down. In certain instances, adjacent embryos would lie side to side in their respective oviducts, while another pair may have their dorsal or ventral surfaces turned toward each other.

One position seems to be assumed by all just prior to birth, however. The offspring shifts about until its ventral and dorsal aspects are the same as the maternal condition. A physical reason for this is simply that the width of the embryo is in the latest stage much greater than the thickness

of the maternal abdomen; therefore the embryo must accommodate itself to the available space.

The nutrient organs

The sources of nutriment must be of interest in an insect with intra-uterine development. Some of the alterations of the mother, embryo, and accessory tissues which serve to convey the nourishment from the one to the other especially deserve our attention. Accordingly, these organs, if they may be so designated, are discussed briefly, with their function as nutrient organs in mind.

Follicular epithelium. The first of these is the follicular epithelium which apparently elaborates and discharges into the lumen of the tubule a product which in Hemimerus is called a fat-like substance by Heymons.

Figure 51 shows a cross-section of the tubule at the posterior end of the germarium or upper end of the vitellarium. The epithelial cells of its walls appear to be filled with a dense but finely granular cytoplasm and they closely resemble glandular cells. The lumen of the tubule appears to contain an excretion from them, as it stains slightly, though this is not an entirely reliable criterion. The wall of the tubule at this point can clearly be distinguished from its more posterior prolongation nearer the oviduct, where it acts perhaps more in the nature of a membrane. It is here about 12 μ in thickness, but lower in the duct it becomes decidedly thinner.

The ovum is less than 15 μ in length shortly after its liberation in the germarium. It at once enlarges rapidly, so that when mature it is eight times greater in size. This increase in bulk is attributed to the active secretion of nutrient solution derived as outlined above.

Nurse cells. During cleavage an accumulation of large cells is found just posterior to the developing egg. These form the second type of nutrient structures and may conveniently be termed the nurse cell body. A portion of such a mass of cells is reproduced in figures 15 and 16.

When first discovered, no interpretation of their function nor their structure could be made, for one of the oldest stages of their history was found and from it nothing could be deduced regarding their past activity.

In their earliest stage they look like slightly enlarged cells with rather large nuclei and a coarsely granular strand-like cytoplasm. Enmeshed in this are many very small, clear vacuoles having the appearance of similar structures in the mature ovum itself. The cell boundaries at first are quite distinctly seen, as are also the nuclear boundaries. In the nuclei are large chromatic bodies which I conclude are nucleoli.

In a late phase of their history one finds the cytoplasm practically absent, having been converted into large, ovate globules throughout the nurse cell body. These stain evenly throughout, revealing no internal organization. In marked contrast to this, the translucent fat-like substance found in the cytoplasmic matrix of the mature ovum does not stain at all. The globules of the nurse cell body are also very much larger than the fat-globules of the ovum; otherwise they appear to be structurally alike—rather, they are both structureless. The nuclei have disintegrated and the chromatic portions of the cells seem to accumulate into long, thick strands, the segmental appearance of which strongly suggests that some physical or chemical property brought them together where they simply adhere to each other rather than fuse (fig. 52).

This progressive process, of course, completely obliterates all cellular organization. The mass rapidly is reduced in amount, presumably through absorption by the embryo, and it soon ceases to exist. Its final disappearance is coincidental with the rapid development of the pleuropodia and their adherence to the embryonic envelopes.

The above sketch of this structure is all that can be recounted because of the scantiness of the material covering this particular stage of embryonic ontogeny. It endures a comparatively brief time, so that quite a series of embryos would be necessary to supply further facts.

The origin of the cells composing this body is entirely a matter of conjecture at present. A solution of the problem has some theoretical value, however, for nurse cells are derived from cells that originally were germ cells. They have lost their function of reproduction. Follicular cells are derived from cells of the ovarian tubule, thus arising as maternal mesoderm.

The typical origin of these cells and their ultimate fate seem not to be true for Hesperoctenes and a different condition appears to exist. Certainly the present function of this cell mass seems to be wholly nutritive. Therefore search of the egg tubule was undertaken in an effort to find such cells, which in Hemiptera are conspicuous and located in the apical end of the ovariole. None could be found above the germ cells. The cells of the nurse cell body did, however, closely resemble the epithelial cells of the anterior end of the vitellarium, both in physical structure and staining qualities, so that this is probably their place of origin. Because of the apparent lack of recognizable nurse cells elsewhere, it was assumed that they must have come from these cells which line the upper end of the vitellarium. As will be recalled, it has already been emphasized that an egg follicle is wanting in this species. In almost all other insects the follicle is typically present. It is always derived from the cells lining the vitellarium (Comstock, '24; Imms, '25). Uichanco ('24) insists that in aphids the egg follicle cells are derived only from follicular epithelium at the anterior end of the vitellarium.

In view of the above unusual conditions of no other discoverable source of nurse cells, yet with an active follicular epithelium from which no egg follicle is delaminated, we may offer this solution. The cell mass may be egg follicle cells originating in the follicular epithelium at the anterior end of the vitellarium. They no longer surround the ovum to secrete the chorion and perhaps to add nutrient substances, as in other insects. Instead they follow after the egg and disintegrate, their remnants being absorbed by other embryonic structures.

To consider the method of incorporating these nurse cells into egg cell substance, we may turn to figures 15 and 16. In the first figure an egg is represented in the second cleavage stage of blastoderm formation with its attendant nutritive body following. Due perhaps to contraction resulting from alcoholic fixation, the line of demarcation between the ovum and its influx of fresh nutriment is clearly discernible. The latter appears as a blunt cone with its base toward the nurse cell body, while its apex penetrates well into the egg cell substance. It is finely vacuolar and less dense as it approaches the egg.

Posterior to this main mass of secretion is a continuation of it in the form of a thin pedicle or stalk which connects with the mass of nutrient cells. It reminds one of the nutrient cords ascribed to amphigonous aphids, but here it is quite short and probably not homologous to the other. The 'nutrient cord' is difficult to see in most sections, appearing but twice in the nutrient masses on my slides. The interpretation that I place upon this structure is that its origin lies in secretions, derived from the nutrient cell mass, which are flowing into the egg. Its shape is due to the contraction of the ovariole, which, being thin-walled, invariably contracts in the absence of ova, embryos, or nurse cells within its lumen. In its contraction the lumen is reduced to the size indicated by the contained nutrient cord connecting the nurse cell body with the egg. The contracted epithelial cells of the tubule are represented by greatly narrowed cells and the nuclei are strongly compressed.

When the blastoderm stage is well advanced, as shown in the second figure referred to above, the nutrient strand still is visible, although not included in this drawing. The constricted tubule is shown as before. The condition of the nutrient cell mass, however, is altered, for it is approaching the earlier stages of dissolution. The cytoplasm is becoming more alveolar. The formation of the large clear ovate globules has not yet been initiated, but they soon will appear. The nuclei no longer show distinct boundaries and the chromatic contents are forming large, conspicuous masses.

Following this stage, complete disintegration of the entire nurse cell body takes place. Therefore it no longer may be said to discharge its contents into the egg cell through the nutrient cord, but from now on and to the end of blastoderm formation we may view the assimilation of additional nutrient material from the nurse cell body as simple absorption of adjacent secretions by the cleavage and germ-band stadia of the embryo

The embryonic membranes. Serosa. When the blastoderm is complete, the egg is surrounded by the cells which compose the serosa. The continuity of the serosa on the ventral surface is interrupted by the germ band, an early stage of the embryo. The details of the changes involved in the formation of the embryonic rudiment in this layer were not found.

Structurally, the serosa appears as shown in figure 22. It consists of a granular and vacuolar cytoplasm with no distinct cell boundaries. It is closely applied to the walls of the ovariole at all times until its period of usefulness is ended. In the drawings, figures 23, 24, and 26, its withdrawal from intimate contact with the maternal tissues is, I believe, a shrinkage phenomenon and does not portray the true situation. In the drawings, too, a narrow space, intentionally emphasized, serves to distinguish between the maternal and embryonic tissues. The nuclei are subglobular, with large and strongly stained chromomeres which appear to be peripheral in position.

Amnion. This envelope appears to result from the invagination of the germ band, for the anterior end of the embryo is directed anad in the maternal body after this membrane is formed. It is very difficult to determine whether the germ band should be classed as superficial or immersed. By consulting figure 21, one realizes the close resemblance of this embryo to the aphid embryo in the position it assumes, but the latter has a large supply of yolk. Due to the fact that practically no nutriment is present in the Hesperoctenes egg, we find the germ band filling the entire cavity, with only the amnion on the ventral side separating it from the serosa. On

the mesoderm side of the germ band the latter is more or less closely in contact with the serosa. The posterior third of the germ band is flexed ventrally, thus drawing this portion of the amnion from the serosa. At this point, too, a small amount of nourishment has accumulated between the two envelopes.

The origin of the amnion, then, is that typical for a large number of insects. Its formation is directly opposite to Hemimerus, as in this insect it arises first, according to Heymons, and from it the serosa is derived later.

The amniotic cytoplasm is comparatively but little thicker than that of many other insects. On the other hand, it does not compare with the extraordinarily thickened amnion of Hemimerus. The nuclei are depressed, so that their apparent length is about three times their thickness. They are rather close together, being separated by intervals of from one to three times the length of the nuclei. The latter are so much thicker than the intervening cytoplasm that they cause the amnion to appear beaded when viewed in section. The chromomeres stain heavily and appear usually to be aggregated at the ends of the nuclei. Parallel strands of linin connect them. This is shown in figure 27.

Both of these membranes suffer the same fate that comes to them in other insects. Just before the destruction of the amnion the nuclei seem to show signs of disintegration through a breaking down of the polar chromatic masses, as seen in figure 53. During the revolution of the embryo they are ruptured and drawn dorsally and forward. After revolution, they may be seen massed just anterior to the embryo. The serosa is gathered into loose folds in the dorsal anterior region of the occiput of the embryo, while the amnion appears occasionally as a thin sheet of cells covering portions of the serosal mass. This is shown in figure 34, which pictures a sagittal section of an embryo shortly after revolution has taken place. During the rupture of the amnion and serosa and their subsequent inclusion in the body cavity of the embryos of many insects, there seems to be a definite contrac-

tion or shrinkage of these cells to form a 'dorsal organ.' This organ is formed of the serosal tissue which in contraction exhibits a more or less cup-shaped mass of cells with the nuclei grouped about it in a somewhat regular arrangement. It has the effect of pulling the membranes into a mass favorable for the overgrowth of the dorsal body wall. The appearance of the dorsal organ is often quite pronounced in insect embryos which must circumscribe a considerable amount of yolk along with the membranes. Such an organ was described and figured in the development of the mantid Paratenodera sinensis (Hagan, '17).

A dorsal organ is not so well developed in Hesperoctenes and the serosal mass appears to lie in loose folds, rather than to be strongly contracted, as one usually finds to be the case. The slight development of the organ and its relation to the membranes as a whole are well shown in figure 35. Its action seems to be confined largely to a minor portion of the serosal mass and the bulk of the latter apparently feels slight, if any, pull by its contraction. The only remaining substances in addition to these membranes now outside the embryo are a few trophocytes, not more than three or four, and one or two small globules of nutriment derived from the nutrient cell mass, though they are almost consumed.

The membranes, being now contracted in the anterior dorsal region, are functionless. Their inclusion within the embryonic body takes place by overgrowth of the dorsal body wall. At a later phase of development they may be found undergoing disintegration within the newly formed mesenteron (fig. 36).

The probable nutrient function of these membranes may now be considered. The close approximation of the serosal envelope to the maternal walls was mentioned. Close scrutiny of the walls of the ovariole showed that their cells as well as those of the serosa are finely vacuolar. The cellular walls of both tissues are very delicate and cannot be distinguished. It seems quite within reason to ascribe an absorptive function to the serosa in the presence of what is apparently an excess

of some substances in the cells of the ovarian tubule. Certainly simple diffusion of nutrient substances must take place from the maternal lymph to the mother's own tissues. The passage of some of it to the serosal layer of the embryo is not impossible, but, on the contrary, is highly probable.

The apparent nutrient function of the serosa seems to be greatly accelerated just prior to the revolution of the embryo. At any rate, the vacuolation is more pronounced and a slight excess of nutrient substance has accumulated. At no time, however, does the serosa send out long cytoplasmic processes to unite it to the maternal tissue as it does in Hemimerus. It depends upon close adherence to the ovarial epithelium at all points and derives its nutriment by osmosis, instead of active destruction of maternal cells. This method of acquiring food by simple diffusion rather than by actual destruction of maternal cells much more closely resembles the true placentation of the Mammalia in this respect than does trophamniotic or trophserosic activity of other insects possessing these structures.

This condition is quite like the situation surrounding the pleuropodia treated below, and further discussion of this question is deferred for the present.

Now to turn to the possible activity of the serosa as a nutrient organ functioning in conjunction with the disintegrating nurse cell body. The independent action of the latter in contributing directly to the egg ceased with the completion of the serosa. There we left it for a time. In describing its fate we found that it continued to disintegrate even after the severance of its connection with the cleavage-stage embryo.

The serosa, being closely applied to the ovariole walls, provided what may be considered an effective plug stopping the passage of the ascending spermatozoa, if they are present, and the downward flow of the products of the disintegrated nurse cell body.

My assumption is that this nutrient material is utilized by the embryo after absorption takes place through the serosal

envelope. Examination of the embryo at this time is necessary; therefore we may again consider figures 23, 24, and 25. It is obvious that there is practically no reserve nutrient material within the serosal cavity. This condition is partially met from another source which in the later germ-band stages provides a slight amount of reserve food in the form of ovate, clear, homogeneous bodies of a nature apparently identical with similar objects mentioned as occurring in the nurse cell body. As I have found them nowhere else in the mother insect, I suspect that they have been derived from the nurse cells. The entire bulk of these masses is rather small and totally insufficient to enable the embryo to carry on its metabolic activities very long unaided by constant additions of new materials. At the mesoderm side of the embryo, however, the trophocytes and migrating mesoderm cells seem to unite the embryonic tissues more or less firmly to the serosa. This condition serves not only to anchor the embryo in place, but perhaps exists primarily to form a source of intake and elaboration of the nutrient solutions to be derived from some agency. That agent can be none other than the serosa, for it alone intervenes between the embryo and the outside food supply.

My whole contention may now be summarized as follows:

1. The embryo has in the amniotic cavity practically no reserve nutriment.

2. The embryo continues to grow and increase in complexity.

3. The serosal envelope separates it from an evident and abundant food supply in the ovarian walls and in the nurse cell body.

The only conclusion to be reached, then, is that the serosa actually does serve as a nutrient organ in somehow passing into its interior nutrient substances as required by the embryo.

This conclusion does no violence to the general rule pertaining to many other viviparous insects, although each has its own peculiar structures involved. Most of them have

in the egg sufficient yolk to bring the embryo to the point of hatching and extrusion from the mother. Hippoboscidae, Glossina, and possibly the Streblidae and Nycteribiidae have this method of development in the egg stage, but the larva is retained in the uterus until ready to pupate. Here they are nourished throughout their larval life by secretions from the 'milk glands' which discharge their pabulum directly into the oral orifice of the larva. Strepsiptera absorb the stored material in the maternal fat-body through permeable egg membranes before the egg yolk is exhausted. Hemimerus depends also on a trophamnion and trophserosa which function throughout the embryonic life of the insect. Just prior to the formation of the trophamnion the insect receives its nourishment through the activity of trophocytes, but even here there is no break in the constancy of nutritional function. In many oviparous species, too, the chorion, serosa, or amnion have trophic functions which are most evident perhaps in certain of the parasitic Hymenoptera (Hill, '23; Leiby, '22). It is believed that in Hesperoctenes the serosa functions for a time as a trophserosa.

Trophocytes. The cells are few in number, but very large (fig. 54). The cytoplasm is always vacuolar, but at times the accumulated products so distend it that it is drawn out into long thin strands to encompass them. The cytoplasm seems to be amoeboid, in that long processes project from it to connect with adjacent tissues. Their nuclei are enormous, filled with numerous small chromomeres and a number of larger chromatic bodies, at least one of which I take to be a nucleolus. Linin threads reach in all directions, knitting the nuclear chromatic granules into a skein.

When they are most active and until the end of their utility, they are found either in the anterior dorsal part of the embryonic haemocoel or in the mesenteron. It is supposed that they finally disintegrate and are absorbed.

Their function is considered to be nutritive, in that they transform food materials into suitable substances for ready assimilation by the embryo. Thus they are present where

the mesoderm of the embryo approaches the serosa. There they presumably serve to convert into nutrient material the substances absorbed by this embryonic membrane and discharged into the serosal cavity. They are also seen in the vicinity of the slight surplus of nutrient material found between the amnion and the serosa. Upon the rupture and contraction of the embryonic envelopes, they are, from then on, in constant association with these now-useless structures. In figure 34 they are already attacking the serosa and in figure 36 they are working over the same material within the mesenteron.

The last of the nutrient organs to be considered are the pleuropodia, which are transient in their appearance in most insects. Their growth and developmental history follows, with some consideration of their probable function.

Pleuropodia. The abdominal appendages arise in the embryo in a manner entirely analogous to those extremities of the preceding body divisions. Their appearance on the germ band lags behind the cephalic and thoracic evaginations and most of them are quite ephemeral. Arising as ectodermal thickenings, they push out, perpendicular to the embryonic body axis, until they become quite prominent subquadrate evaginations. From the first their interior is invaded by a mass of mesodermal cells which quickly assume the shape of a more or less cone-shaped core with a hollow center. The interior mesodermal cavity could not be traced to a dorsally situated coelomic rudiment, but seems to extend to the mesoderm of the future haemocoel.

Of these abdominal evaginations, the anterior ones seem to be always in advance of those more posteriorly situated, both in time of appearance and size. In fact, disregarding some exceptions not pertinent to this paper, the others soon subside to the general level of the ectoderm, where they are thought to form the lateral margins of the sternites.

The germ-band stage. But this is not the history nor the fate of the pleuropodia or appendages of the first abdominal segment of Hesperoctenes. In their earliest evagination they

appear quite like the thoracic limb buds and similar to the latter in position on the germ band. They differ from them only in being slightly shorter in length. While they were simple swellings of the germ band few mesoblast cells were noticeable in them, but with the slightest outward growth these cells begin to form a very definite mass in the interior.

The youngest embryos bearing pleuropodial structures are represented in figures 20 and 21. The first shows only the maxillary and succeeding segmentation, the anterior portion being in other sections. It may be seen that segmentation extends only to the first four abdominal segments, the posterior portion remaining undifferentiated as yet. The second figure represents the entire embryo with segmentation practically complete. The approximate length of the embryo in this stage is 0.7 mm., while the pleuropodial rudiments are 0.035 mm. in length.

Early embryonic stage. In an older embryo in which limbs have elongated and the prothoracic pair, at least, have turned anad, they seem to be quite limb-like when comparing their development with that of the prothoracic and metathoracic limbs (figs. 23, 24, and 25). Their continued growth causes them to project outward from the abdominal wall until they press against the telson, which appears opposite them at this time. The posterior half of the abdomen still is flexed ventrally, so that the tip of the abdomen extends forward almost to the thoracic region. Only the amnion separates the first abdominal appendages from the telson. The pleuropodia at this evaginate stage measured 0.05 mm. in length and 0.04 mm. in width. The length is only approximate, as the appendages have been distorted somewhat by pressure against the opposing telson. The ectoderm is composed of cells quite like those of other parts of the body. Cell boundaries are not distinguishable in the specimens studied and the nuclei possess no characteristic features to set them apart from the general ectodermal nuclei (fig. 29).

Turning for a moment to a consideration of the pleuropodia in Belostoma and Ranatra, which are insects within the order

Hemiptera, to which Hesperoctenes belongs, Hussey ('26) recently has described them as projecting about half their length above the ectodermal surface. They never contain mesoblast cells. Their cells become very elongate and finally sink beneath the adjacent ectoderm. Here they become globular or subglobular invaginations, with perhaps some glandular activity. The projecting attenuated tips of their cells, brush-like, persist until hatching. These may, in fact, extend outward sufficiently far to come in contact with the thoracic appendages which are directed posteriorly before them.

According to her, also, somewhat similar observations have been made by Heymons on other hemipteran species, among them Naucoris cimicoides L. and Cimex dissimilis Fabr.

In none of these insects do the pleuropodia evaginate as such massive structures as they do in Hesperoctenes nor do they play so important a part in their invaginated later stages.

Comparing the development of these organs in the preceding insects with Hesperoctenes fumarius, we find that a quite similar history has been followed by all of them. Evagination, followed by invagination later, is not peculiar to these insects alone, but is common to several other species. Hussey, however, recently has attempted to trace in detail the steps involved in the process and to find the intermediate steps between the evaginate and invaginate stages.

No further comparisons between her specimens and Hesperoctenes can be made, because these steps unfortunately are lacking in my material, but without doubt the process must be similar and the end product is in general identical in the three examples, namely, an invaginated globular or subspherical mass of enlarged ectodermal cells forming the pleuropodium with the distal tips of the cells projecting brush-like from the embryo.

Prerevolution stage. This procedure of invagination is but recently completed in the specimen shown in cross-section

in figure 30. The section was not truly a cross-section at right angles to the body, as it will be seen that only one pleuropodium was sectioned. On the left the posterior margin of the metathoracic limb appears. This results in the illusion that the pleuropodium really is longer than actually is the case, for it is cut in a slightly diagonal direction. The embryo is in the stage just prior to revolution. The amnion and serosa both are present, but the latter is withdrawn from its former intimate contact with the wall of the ovariole. This may be due to shrinkage, of course, and this possibility must be taken into consideration.

In this stage we may see a series of changes. While the ectodermal cells of the ventral body wall have become a definite, narrow layer of hypoderm, the pleuropodial cells, on the contrary, have enlarged enormously. The individual cell shape is conical, elongate, with fine cytoplasmic processes extending outward into the amniotic cavity. The cytoplasm is finely granular, and cell boundaries can be made out only as the result of shrinkage, or oblique lighting occasionally reveals them. Cell walls cannot be distinguished. Distally, the cell tips seem to approach the amnion, which in this particular specimen could not be seen directly opposite them. It may actually have been absent, due to disintegration, although present elsewhere. The serosa also seemed to be undergoing dissolution at this point. At the extremities of the pleuropodial cell tips are found small, round or oval masses of material indistinguishable from the pleuropodial cytoplasm except that optically they seem to stain somewhat more intensely. These may be anterior or posterior extensions of the severed pleuropodia or may be secretions. Similar observations have been made previously by others investigating the pleuropodia of various species of insects. They have invariably been termed secretions or excretions.

The inner portion of the pleuropodium is more sharply defined. It contains the major portion of the cell bodies and their nuclei. Altogether they form a very large subglobular mass lying dorsolaterally to the nervous system and occupy-

ing all of the dorsal region of the embryo. Indeed, their inner margins are in contact in this stage. Laterally, the pleuropodial cells merge gradually into the typical ectodermal layer. The cell nuclei are very large, with clear nucleoplasm. In the interior chromomeres and prominent nucleoli are plainly visible.

Many other insects have pleuropodial development quite like the preceding stages just described. Some indeed, as in Orthoptera, show them much more highly differentiated into limb-like organs in evaginate stages. Others of the invaginate types, as Dytiscus, may have the pleuropodial cell tips project outward quite far, even touching the amnion, but none become so massive or occupy so much space in the embryo as they do in Hesperoctenes.

Postrevolution stage. During revolution of the embryo the amniotic and serosal membranes are ruptured and massed cephalad, where they may be seen lying in folds adjacent to the occiput. The initial steps in the destruction of these envelopes may have commenced in the preceding stage through the activity of the pleuropodial cell tips as already suggested. In other insect species without such excessive pleuropodial development a similar treatment of the amnion and serosa is found, so that their secretions or adherence may have nothing to do with it. Following the dorsal retreat of these membranes, however, the pleuropodia rapidly extend their own cell tips. Most rapid growth seems to be in an anteroposterior direction, and lateral extension is somewhat slower. This condition is found in a longitudinal section of an embryo which has just completed revolution, whose degree of development is shown in figure 34. The pleuropodial cell tips reach almost to the head and tip of the abdomen, but no trace of them could be found along the dorsal and ventral surfaces of the embryo. Between the time of the contraction of the embryonic membranes and the completion of circumscription of the embryo by the pleuropodia, it must be free in the lumen of the oviduct without embryonic covering. During revolution the embryonal abdominal region straightens

out or is only very slightly ventrally curved, as in other insects.

The same drawing shows the severed inner portion of the pleuropodium in cross-section. Other views have been cross-sections of the embryo, but longitudinal sections of the invaginated portion of the pleuropodia. The interior parts of the pleuropodia actually are about one-fourth or one-fifth the body length of the embryo and take up most of the space within the body cavity. The only organ that approaches it in this respect is the central nervous system. The diameter of the organ at this stage measures 0.15 mm., but in the figure shown the greatest diameter is 0.10 mm., for this is not its widest portion. The dorsal wall of the embryo encloses it in a definite body cavity with the scattered trophocytes, mesodermal cells, and fat-body cells. The amnion and serosa gathered anteriorly are not yet invested.

The embryo shows a complete absence of food material except for the embryonic membranes, which, of course, will be utilized and probably are being digested. Indeed, in the absence of another source of supply, the trophocytes must elaborate from these membranes sufficient amounts to satisfy present needs that undoubtedly exist, to nourish the embryo in its development and to enable the pleuropodia to complete their extensive external growth.

In figure 31 is shown a cross-section of the embryo after the pleuropodia have surrounded it. The dorsal body wall is formed and the mesenteron is complete. The labium, antennae, and legs are directed anad. The metathoracic appendages almost reach the tip of the abdomen.

While the pleuropodia are as large as in the previous stage, yet they are now separated from each other internally. This comes about through the completion of the dorsal body wall, the beginnings of differentiation of internal mesodermic structures and the mesenteron. The embryo, too, has broadened considerably. In the figure shown the asymmetric curvature of the body is due to the pressure of adjacent embryos in other ovarioles or oviducts.

In subsequent stages it appears that the pleuropodia continue to occupy a large part of the haemocoel immediately adjacent to the mesenteron. Their unusual size must cause them to crowd other organs and would appear to be entirely superfluous unless their presence is correlated with some important task.

The cell tips of the pleuropodia can be seen, as in the pre-revolution stage, extending like fibrillae into the lumen of the oviduct. The lateral, forward, and back extensions of them do not, however, appear thread-like. This may be due to several causes. Those that occur to me are: *a*) that the extensions may not be cellular substance at all; *b*) sectioning may not be made in a plane parallel to the cell walls; *c*) the extensions may be truly cytoplasmic, but the cell walls may be wanting or so extremely tenuous as to be invisible. In the light of the probable function of these organs, to be discussed later, the last interpretation appears to be the only tenable one. To support it, too, may be mentioned the fact that the entire structure stains alike and in numerous other sections no cellular boundaries can be seen except in one specimen represented in figure 55.

The embryo referred to recently has been completely enclosed by the pleuropodial structures. The section was cut diagonally in such a way that it apparently followed the line of growth of the cellular extensions to their points of contact with the wall of the oviduct. This gives the nuclei an elongate appearance which greatly exaggerates their longitudinal axis.

In examining this pleuropodium, which, by the way, is represented in the figure as a composite drawing obtained from two sections, one is able to trace cells directly to their apparent contact with the wall of the oviduct. The distinctness of the parts probably is due to the shrinkage suffered by these insects when dropped into alcohol. Their resulting relationships with one another and with adjoining tissues should be subject to later verification.

As the pleuropodial cell tips approached the walls of the oviduct they seemed to become faint pink in color and highly vacuolar. Contact with the walls actually occurred, but the line of demarcation between the two tissues remained distinct. The oviduct epithelium was composed of cells whose cytoplasm was even more alveolar than the pleuropodial cells and they stained slightly lighter in color as a result of this condition.

But this does not give one a conception of the pleuropodial extensions as a whole. After enveloping the embryo, the margins of the right and left pleuropodia fuse. No line of demarcation serves to identify either pleuropodium and they appear as a common mass of material. The embryo now is enclosed in a new embryonic membrane not formed at all of blastodermic cells or material antedating itself in time, but it is surrounded by a sheath supplied by an extension of its own ectoderm. This is thought to be unique in insect embryology. Outside of this sheath is the lumen of the oviduct, while the space enclosed and containing the embryo may well be called the *pleuropodial cavity.*

Shortly after completion of the pleuropodial growth, their extended portion may be seen to be constantly in contact with the walls of the ovarian tubule or oviduct. This follows naturally, for the elastic walls of the reproductive organs are dilated by the bulk of the contained embryos. Shrinkage in my material shows little of this intimate contact in its proper relationship, but attention is called to the probability that such a condition is normal.

While such contact may serve useful functions, as food absorption, discharge of wastes, and possibly others as well, it must be recognized that serious difficulties present themselves which apparently conflict with the normal course of other activities. The contact cannot be too close a union or the embryo could not shift its position to lower levels in the oviduct. Without doubt it would interfere with the upward migration of spermatozoa should they attempt to pass the embryo and would serve to check or prohibit the flow of nutrient materials, if they are present, to the embryo below.

The distal parts of the pleuropodial extensions do not exhibit cellular organization and the nuclei are all found lying within the basal portion of the organs. Sections of the pleuropodium at points *x* and *y* in figure 31 are shown enlarged in figures 32 and 33. The matrix is composed of finely granular material and resembles cytoplasm in physical appearance. Some physiological activity is present, for one may distinguish a number of small vacuoles embedded in it. They are more numerous near the embryonic or inner half of the pleuropodial extension and frequently approach the margin. It is entirely possible that the contents of these vacuoles are discharged within the pleuropodial cavity. This is a function of certain kinds of epithelium. The acquirement of a cuticula on the outer surface of the pleuropodia in later embryonic life, as described below, should convince one that the material composing the pleuropodial extensions is truly cytoplasmic in nature. This closes my comments regarding the interpretation of these peculiar organs as cytoplasmic extensions of the pleuropodial cells.

Cuticular stage. In this stage the hypoderm of the embryo has secreted a cuticular layer. The embryo is found in the uterus or common oviduct, but it has not yet assumed its final position for extrusion. Generally, it is lying upon its side. Its length is almost that of the maternal abdomen. The pleuropodia still enclose the embryo, but the cytoplasmic extensions seem to be thinner than in earlier stages. The invaginated portion is 0.09 mm. long and 0.11 mm. wide (fig. 56). It appears to be relatively quite small compared with the entire bulk of the embryo and it no longer protrudes far into the body cavity, but occupies a lateral position and is partially surrounded by fat-body cells.

Closer examination shows that posteriorly the pleuropodial extension seems to be invested with a cuticular layer, though in this stage it does not extend to the first abdominal segment. Such a covering would preclude the possibility of cytoplasmic contact between the pleuropodia and the wall of the uterus. Slightly later embryos show this secretion in the anterior

abdominal region as well. A section of this organ at the posterior end of the embryo is illustrated in figure 57. It shows the cuticula as a very thin, limiting, structureless membrane. Within it the pleuropodial extension seems to be divided into three fairly definite layers. The outer one, adjacent to the cuticula, is densely and finely granular, the middle layer is far less granular and therefore appears lighter in color, while the innermost is composed of round or oval masses of substance. The last may be either secretion, or more likely, the disintegrating pleuropodium itself.

The invaginated portions of the pleuropodia also offer points of interest to us. The nuclei seem to be unchanged for the most part. Occasional chromatic bodies to be found for the first time in this organ, however, strongly suggest that some of the nuclei are breaking down. This is confirmed when attention is drawn to the general shape of the organs. They have lost their rounded form and have become rather irregular in outline. The cytoplasm is highly vacuolar, with only a small proportion of the former amount left. One, in observing them, must conclude that the functional service of these organs is largely over.

Stage prior to hatching. This stage refers to the oldest embryo found. The organs are all formed and most of them are ready to function. Birth of the embryo is imminent. The invaginated portion of the pleuropodium is much shrunken and almost devoid of cytoplasm. Measurements show it to be only 0.07 mm. in length and 0.04 mm. in width. The description given immediately preceding showed it to be broader than long; now its proportions are reversed. It lies closely in contact with the body wall, which, in this specimen at least, seemed to be flattened into a pleural ridge at this place. There is a conspicuous absence of mesodermic tissue adjacent to it. The nuclei actually are smaller and they, too, show signs of deterioration. No longer are they to be found only at the bases of their cells, but in some instances have slipped forward toward the central portion of the organ (fig. 58).

Of the pleuropodial extensions, nothing remains but the cuticular layer. The cytoplasmic part has entirely broken down and disappeared, probably through absorption within the embryonic body.

This description of the pleuropodia leads us to a consideration of their probable function in Hesperoctenes. It seems incredible that such a marked development of useless organs would persist.

Function of the pleuropodia. The origin of the pleuropodia in insects shows that they are evidently embryonic vestiges of former functional appendages. The probability is increased in one's mind when homologies of the appendages are studied in the light of their phylogenetic significance. This conclusion was reached some years ago by the earlier students of arthropod embryology following the discovery of pleuropodia by Rathke in 1844. Not all are agreed, however, as to the original function of these appendages. Hussey ('26) has compiled records of pleuropodia in forty-six species representing thirty-two families and fourteen orders. Writers have ascribed functions to twenty-one species.

In these twenty-one species whose pleuropodia are said to possess some activity the respiratory function was suggested by Rathke for Gryllotalpa, by Ayers for Oecanthus, and by Baillon for Meconema. A sensory function was ascribed to them by Patten, who worked with Gryllotalpa. The remainder agree that the pleuropodia possess a glandular function.

Apparently, Patten was also the first to suggest the glandular nature of these appendages. Wheeler advocated this view, and the appearance of his paper was followed the next month by one from Graber also recognizing their glandular nature.

Wheeler's ('90) thorough analysis of the other theories and careful presentation of his own views on the glandular hypothesis leave nothing new to offer at the present time. In this regard one of his statements ('90, p. 113) is especially stimulating, for he says: "In view of the ectodermic origin

of the pleuropodia, they may be said to have had one of three functions; they were either respiratory organs, sense organs or glands." Hesperoctenes fumarius probably is simply another species whose pleuropodia have had a secretory function.

It is not within the province of this paper to attempt to account for the former use to which these appendages were put, but rather to offer some explanation for their present enormous enlargement and possible function. My conclusion is that they serve at present as nutrient organs in Hesperoctenes fumarius. Subsidiary to this thesis are two additional possibilities: they may serve for the exchange of gases, principally oxygen and carbon dioxide, and also they may excrete liquid waste products.

It is a function of epithelial tissue to absorb substances, on the one hand, and to discharge them as secretions into another element or situation. This is the process by which the endothelium of the hexapod elaborates the food in the alimentary tract and passes it into the haemocoel. The reverse steps occur when the epithelial cells of the salivary glands discharge their secretions into the lumen of their ducts and when the hypoderm secretes the cuticula.

The plasticity of epithelial tissue is well known. It is the buffer between the animal and its environment and thus must exhibit considerable adaptability. But this pleuropodial tissue, being embryonic, may be expected to be particularly susceptible to an alteration of function. Other tissues, likewise, may be able to take up tasks not originally assigned to them, but nevertheless existing as potential functions, needing only a proper environment for their expression. Coelomic pregnancy among the higher animals, the absorptive capacity of the epithelium of cestodes, the trophamnion in several species of insects, the trophserosa and trophamnion in Hemimerus, and the ectodermal intake of food by Strepsiptera from the host's tissue are examples of this ability of tissues to change the nature of their labor under new conditions.

In addition to the above points and, indeed, presenting the whole subject of the secretory ability of the pleuropodia are Wheeler's statements ('90, pp. 116, 117) so clearly set forth in support of their glandular function. The fact that they were written to support the theory that their original function in the past was glandular does not alter their importance here in the least. They seem to apply with equal force to the present condition found in Hesperoctenes; he says, in part:

> The following are my reasons for assigning a glandular function to the pleuropodia.
>
> 1. The entire ectoderm of Arthropods, excepting its nervous derivatives, is essentially a glandular layer, one of its prime functions being the secretion of the chitinous armour so characteristic of these animals. This function is retained by the ectoderm cells, even when they are pushed into the body as in the case of the tracheae, tentorium, oesophagus and rectum.
>
> 2. The pleuropodial cells closely resemble other simple ductless glands in insects, such as the wax-glands of the Aphididae and the stinging glands of some Lepidopterous larvae.
>
> 3. The pleuropodial cells in several insect embryos produce a secretion, the character of which differs considerably in different forms.
>
> 4. In some insects at least (Blatta, Periplaneta, Xiphidium, Stenobothrus) the chitinous cuticle does not cover the pleuropodia, even after investing the body of the embryo. On the supposition that we are dealing with ductless glandular organs, the reason for this is obvious. The secretions of cutaneous glands cannot penetrate a thick and unmodified layer of chitin, so we find gland cells covered with a cuticle the (chemical?) structure of which departs from that of ordinary chitin.
>
> 5. The lack of any apparent innervation to the pleuropodia, though adducible as a fact against the sensory nature of the organs, is just what we should expect on the supposition that they are glandular.
>
> 6. The manner in which some pleuropodia degenerate suggests what is known to take place in many glands that indicate their relation to epithelial structures by secreting their own broken-down cells.
>
> 7. The structure of the pleuropodia described up to the present, though considerably diversified, is in all cases consistent with a glandular function.

The evidence appears to be sufficient, then, to justify the supposition that these structures may quite logically assume the rôle of nutrient organs without violating our conception of their possibilities.

Now to turn specifically to other conditions existing in Hesperoctenes embryos and their environment which may support the contention that the pleuropodia are nutrient in function.

1. The embryo lacks the yolk normally present in the other insects except Hemimerus. This is the most striking impression that one receives in the entire study of this insect's embryonic history.

2. No other form of stored nutriment is apparent and available to the embryo.

3. The serosa, which in the germ-band and early embryonic stages appeared to function as a trophserosa, is no longer functional after the initiation of revolution. No other organ besides the pleuropodia can be seen to take its place as a nutrient organ. It might be claimed, however, that the serosa already was sufficiently voluminous to supply food for the embryo until its extrusion. This is doubtful, as the bulk of the developing insect certainly increases much greater in amount than can be accounted for by conversion of the embryonic membranes alone.

4. The pleuropodial extensions surround the embryo shortly after revolution and entirely separate it from outside sources of nourishment unless such nutrient material passes through the pleuropodial structures.

5. The trophocytes are the only other structures that appear in this embryo which might be credited with nutrient ability. They, however, always are enclosed within the serosa or embryonic ectoderm and thus have available only material that comes to them first through serosal activity or later by permeation of the pleuropodia. It should be pointed out, however, that they do cause the destruction of the embryonic membranes after dorsal closure.

6. The internal portions of the pleuropodia occupy a strategic position favorable to their action of nutrient organs. From them nutrient secretions could as readily be distributed as from the mesenteron. Their large size, too, offers ample surface for the discharge of their contents.

7. The distal portions of the pleuropodia seem to come into close contact with the walls of the ovarian tubule or oviduct.

8. The lack of cell walls in the pleuropodial extensions or their extreme delicacy would favor ready osmotic acquirement of food materials.

9. The walls of the reproductive system are finely and profusely vacuolar, indicating an excess of nutrient or other substances. This is understandable from the very nature of the internal anatomy of insects where the reproductive system lies in the haemocoel and is bathed in nutrient-saturated lymph.

10. The walls of the reproductive system consist apparently of a single epithelial layer, a basement membrane, and perhaps an investment of a very delicate epithelial tissue. I find no muscular coats. Ready diffusion of nutrient substances through these walls easily could take place by osmosis.

As a corollary to ascribing a nutrient function to the walls of the reproductive system by osmosis—and osmosis is a common phenomenon when concentrates are separated from more dilute solutions by a permeable membrane or tissue—we can also assume that the same operation holds true for the products of metabolism—liquids and waste gases. This operation appears much more difficult to account for in the case of an embryo enclosed in a chorion than one encased in a tissue. There is no chorion present in Hesperoctenes.

In closing this discussion of the pleuropodia it might be desirable to call attention to the present lack of a suitable term to cover these organs in Hesperoctenes and the corresponding structures in Hemimerus. None has as yet been proposed for the first genus, but Heymons referred to the nutrient organs of Hemimerus as placental organs. At the same time he recognized the inadequacy of the term, which

properly is applied only to a vertebrate organ. There is, of course, no homology between the vertebrate and invertebrate structures under consideration. Heymons ('09) remarks that they simply have analogous functions, which I regard as true also for Hesperoctenes.

To designate these nutritive structures in Hemimerus and Hesperoctenes, it is proposed that the term 'pseudoplacenta' be employed, as this word suggests the nutrient function, yet makes no claim of being a homologue of the placenta.

Nutrition and the migration of the embryo. Having now given a résumé of the embryonic development of Hesperoctenes and the probable sources of its nutriment, it might be well to inquire into another phase of this problem. I refer to the migration of the embryo down the reproductive tract and its possible relation to the first two factors.

The embryo begins as a zygote near the apical end of the ovariole. It migrates down the ovarian tubule into one of the paired oviducts, thence into the common oviduct or vagina, and eventually is born.

During this journey the embryo also must change its position three times in relation to the maternal reproductive system. First, as a germ band it reverses its orientation and comes to lie with its cephalic region directed down the ovariole. Next it undergoes revolution whereby it again shifts its position so that its posterior end precedes the head portion. Here we find it lying on its side. The third adjustment comes about as it accommodates itself to the abdominal space it has to occupy. In this movement it finally comes to lie with its ventral surface toward the ventral aspect of the mother.

Its first change results in the formation of the amnion. It is interesting to observe that with the formation of the amnion during the first shifting, the serosa is forced to begin the nutrient function as the germ band is entirely enclosed by it. The second revolution destroys both amnion and serosa, and the pleuropodia happily encompass the embryo and nourish it. At the third shift to bring the dorsoventral surfaces in the same position as the mother's the nutritional function of the pleuropodia is over. Birth follows quickly.

From this we see that during the migration of the embryo it has developed, utilized, and destroyed these structures very much as other insects do in their embryonic history. In addition, Hesperoctenes somehow has been able to use two of these structures as nutrient organs where most other insects do not. Other exceptions to the general rule, including Hemimerus, Strepsiptera, and some Hymenoptera, also use absorbing structures simply because the egg chorion is permeable or absent, thus permitting such an accommodation to the environment. Evidently, then, the possibility of absorptive function may be potential in all hexapod serosa.

In this connection we have an explanation why placentation could be only transitory and lack the firmly attached, parasitic type of growth customary to the mammals. Differences in food and air transport and so on, between the haemocoelic insectan type and the closed circulation of the mammal, are beyond the scope of this paper and are well known to embryologists. One of the main reasons for these rather superficial serosal and pleuropodial attachments must be that these membranes are shortly to be destroyed and replaced by others—thus their duration is brief. But more important still is the process of migration. As each embryo must continually give way to the embryos above, the connection of its membranes to the maternal tissues is constantly in a process of readjustment in relation to its new position farther down the reproductive tubule. It follows that if too close a union occurred each time, interruption of the connection would result in frequent and perhaps severe injury to the parental or embryonic tissues. Osmotic exchanges of plain surfaces do not necessarily involve more than simple adhesion of tissues. This is unlike Hemimerus, for in the latter the maternal and filial tissues are so closely knit by the fusion of cellular elements that severance of the relationship is impossible. As a result, the parasitic offspring actually destroys the parental follicle pseudoplacenta and the egg follicle cells.

The rate of migration also is of interest. If we assume that ten to twelve eggs or embryos are present at one time

in the reproductive system, obviously the rate of migration of the youngest ovum would suffice to bring it to the vagina, a completely formed insect ready for birth. Its rate of migration then exactly corresponds to its embryonic history. This is quite the opposite to Hemimerus, which has no migration until its entire embryonic life is finished, whence migration results at once in birth.

The manner of this downward progression of the embryo probably cannot be determined. It is possible that the movement is gradual and continuous on the part of the embryo, and thus we see a mechanical preventative of the complete fusion of the embryonic and maternal tissues. However, when we note the relative sizes of the embryos and recollect their individual rather than simultaneous births, we may conceive of them as moving down in a series of steps or a succession of short migrations alternating with periods of rest. The latter ensue until another birth permits again a slight descent of each of those above.

SUMMARY

The literature relating to viviparous reproduction in insects is enormous. Others have been content merely to accept the cases recounted and in some instances have tried to explain the presence of viviparity through the influence of environment in the acquirement of new characters or have called upon natural selection to account for their origin. In the past only one writer has made any serious attempt to classify the viviparous forms according to the conditions surrounding such births.

The classification of viviparity proposed herein is an effort to recognize the importance of the interrelationships existing between the parent and her offspring during the embryonic development of the latter. It is obvious that this aspect of viviparity is the critical difference between the viviparous type of birth and the oviparous condition.

The proposed classification is based upon four essential differences: viviparity with no nutritional structures, vivi-

parity with nutritional structures for the larva only, viviparity following haemocoelous development and fat-body absorption, and, finally, viviparity following the development of pseudoplacental organs.

The first three types of viviparity were illustrated briefly by examples of insects already appearing in the literature, and the bulk of this paper has been devoted to the exposition of the fourth type. Heretofore one insect, the rare Hemimerus talpoides Walker, remained unique among insects in its embryonic development. No one has attempted to classify its type of viviparity.

Having presented this fourth type of viviparity, its permanent position is assured by the presence in it of Hemimerus and an even more remarkable example in Hesperoctenes fumarius Westwood whose embryogeny is summarized below.

The maternal abdomen of Hesperoctenes fumarius is largely given over to the incubation of the embryos, and the mother's organs are pushed aside by the developing offspring. Apparently about ten of the latter constantly are present in the mother, in stages varying from ova to embryos almost ready for extrusion.

The method of impregnation of the female is unknown, but three possibilities are suggested, with comments upon each. Spermatozoa are found within the maternal haemocoel lying in clumps of two sorts: one, a compact cylindrical mass in which the spermatozoa are all oriented alike; the other, a loose aggregation of spermatozoa with no symmetrical arrangement. Spermatozoa also may be seen in the tissues of the female reproductive system. To secure the union of the biparental elements, the sperm cells must penetrate the ovarian tubule to its lumen. Fertilization occurs immediately below the germarium.

The first mating of the female and her impregnation with spermatozoa take place prior to her maturity. This is most extraordinary in insects.

Offspring begin their development before the mother assumes physical maturity. While the young may be born

after the attainment of maturity by the mother, nevertheless her firstborn, perhaps four to six in number, must be considered as examples of paedogenetic reproduction.

Previous examples of paedogenesis in insects have followed the parthenogenetic condition. Hesperoctenes differs in being the first recorded case of bisexual paedogenesis in this class.

Uichanco's classification of paedogenetic forms has been revised in order to admit this species.

The female reproductive system consists of two ovarioles and an oviduct on each side of the body. The latter unite to form a short, unpaired oviduct which opens to the exterior.

The ovum is unusual in being yolk-free. It is furnished with fat-globules sufficient in amount to initiate development. No chorion, or eggshell, surrounds it, for no egg follicle cells envelop it.

Cleavage is complete, each blastomere lying in the periplasmic region. The process of segmentation and the appearance of the extremities appear to be that typical for related insects. The exceptional point is the continued growth and persistence of the pleuropodia, or appendages of the first abdominal segment. They evaginate quite like the thoracic limbs.

About the time of revolution of the embryo the pleuropodia become invaginate and extend into the embryo until their inner ends touch each other.

After blastokinesis, the embryo rapidly approaches the definitive nymphal type. The embryonic membranes are enclosed within the mesenteron, and the fusion of the dorsal body walls takes place. The pleuropodia, now invaginated, extend their cell tips to enclose the embryo in a cytoplasmic sheath which I term the pleuropodial extensions, and the space enclosed is called the pleuropodial cavity.

From the initiation of blastokinesis until the completion of the pleuropodial extensions the embryo lies free in the maternal reproductive tract without attachment to the mother and without any protective covering. This condition is believed not to occur in any other embryonic history.

The embryonic hypoderm secretes a chitinous exoskeleton prior to birth with the full nymphal armature of setae. The pleuropodial extensions likewise secrete a cuticula which serves as an embryonic envelope during extrusion.

The nutrient organs are remarkable in their number. The fat-like substance which accumulates in the egg comes from the epithelium of the tubule. The nurse cell body seems to be derived from the follicular epithelium, the usual source of the egg follicle. The trophserosa develops from the blastoderm and absorbs the nurse cell nutrients. The trophocytes elaborate in succession the substances available to them within the serosa, the pleuropodial cavity, and the mesenteron. Finally, the pleuropodia take over this function and continue to supply the embryo with nutriment until shortly before its extrusion from the mother. In so doing they function in a manner analogous to the placenta of the mammals. Not being homologous with the latter, however, I have proposed that such organs in insects, whether pleuropodia or others of similar function, be termed pseudoplacenta.

The pseudoplacenta of Hemimerus is firmly attached and the embryo remains in one location within the ovarial tubule until ready for birth. Hesperoctenes migrates down the tubule as it secures nourishment and develops. This precludes such intimate union with the maternal reproductive system.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

1 Composite, semidiagrammatic drawing of sagittal section of nymph in pre-adult stadium. Head and prothorax not shown. × 95.

2 Semidiagrammatic drawing of longitudinal section of nymph in pre-adult stadium. Head and prothorax not shown. × 95.

ABBREVIATIONS

an, anus
c, central nervous system
c3, thoracic ganglion
c4, abdominal ganglion
co, colon
cov, common oviduct
cu, cuticula
cul, loosened cuticula
f, fat-body cells
h, hypoderm, ectoderm
hb, pericardial cells
he, haemocoel
hi, hind intestine
me, mesenteron
ml, malpighian tubule
o, maternal genital duct
ov, ovum, or oocyte
pov, paired oviducts
s, spermatozoa
tm, thoracic muscles
tr, trachea

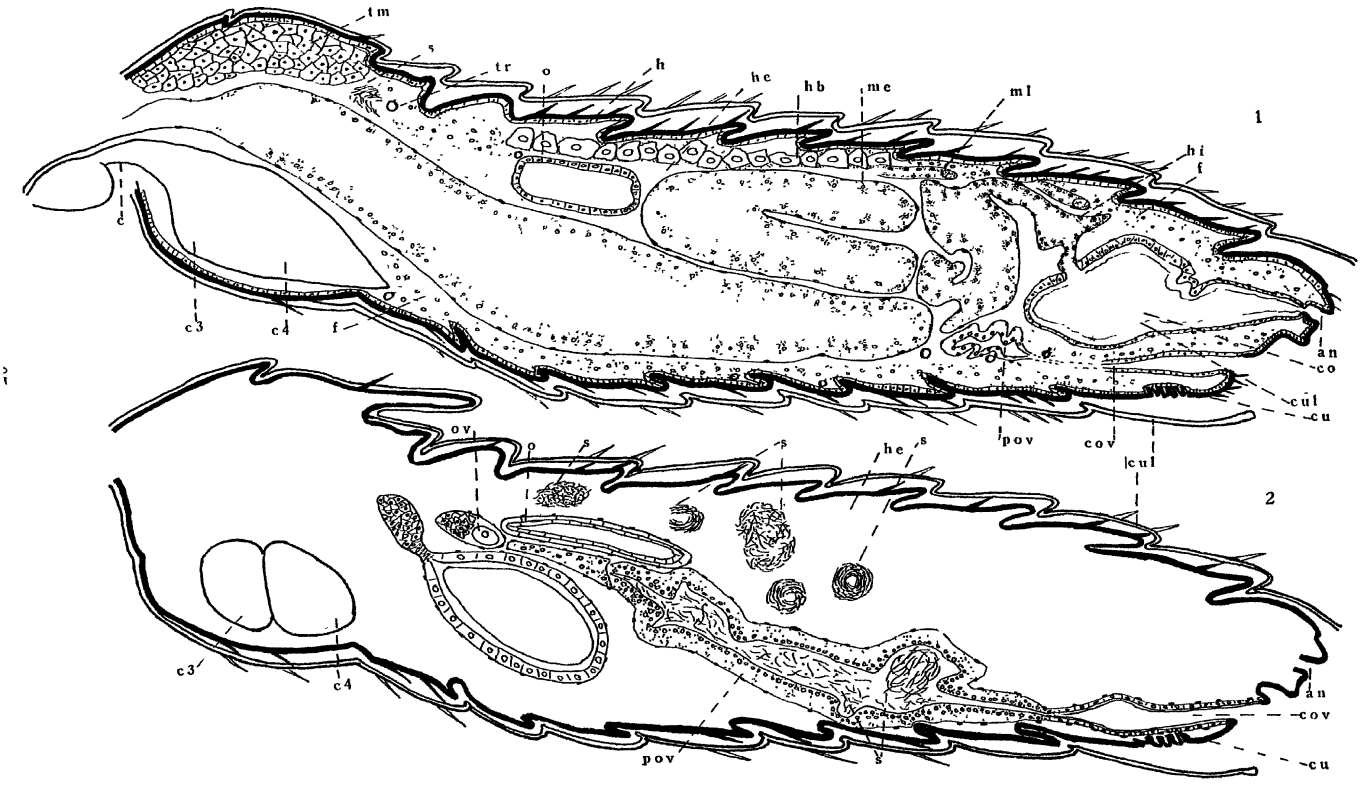
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PLATE 2

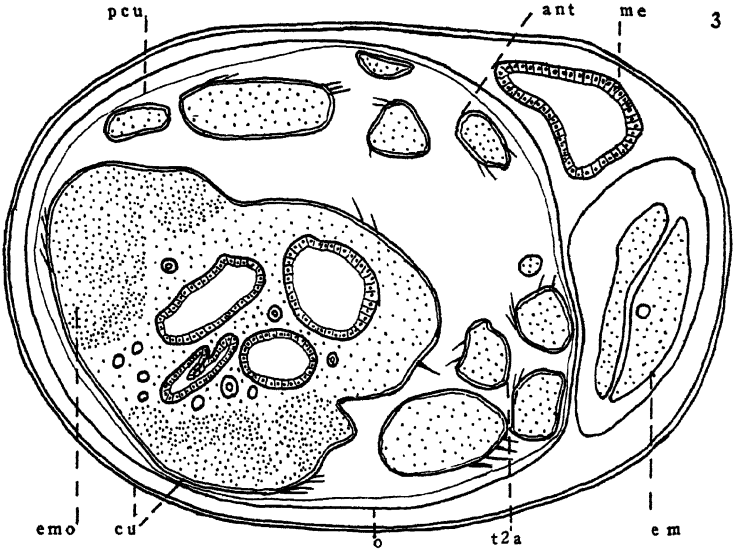
EXPLANATION OF FIGURES

3 Diagrammatic cross-section of maternal abdomen to show area occupied by older embryos. × 100.

4 Diagrammatic longitudinal section of maternal body to show space occupied by older embryos. The second oldest embryo is omitted, as its position parallels the oldest one and would obscure the latter, if included. × 50.

ABBREVIATIONS

ant, antenna
cu, cuticula
em, embryo
emo, chitinized embryo
me, mesenteron
mt, mesothorax
o, maternal genital duct
p, pleuropodium
pcu, cuticular secretion of pleuropodium
t2a, mesothoracic appendage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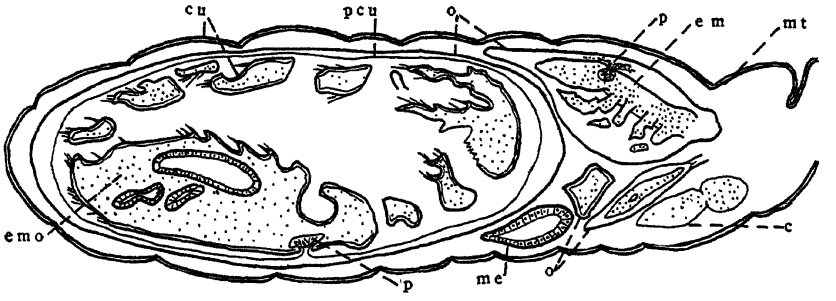
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PLATE 3

EXPLANATION OF FIGURES

5 Cross-section of apex of germarium. × 1600.

6 Longitudinal section of germarium. × 760.

7 Section of germinal epithelium of germarium, greatly enlarged. Free-hand drawing.

8 Follicular epithelium of figure 6, greatly enlarged. Free-hand drawing.

9 Cell of oviduct. × 1600.

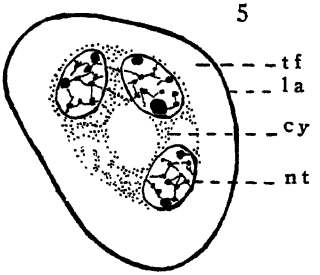
10 Nucleus of another cell of oviduct. × 1600.

11 Germarium containing very small oocyte. Compare with figures 6 and 12. × 760.

ABBREVIATIONS

cy, cytoplasm
g, germ cell
la, lamella of terminal filament
n, nucleus
nc, nurse cell
nt, transverse cells at apex of germarium
ov, ovum or oocyte
tf, terminal filament

5



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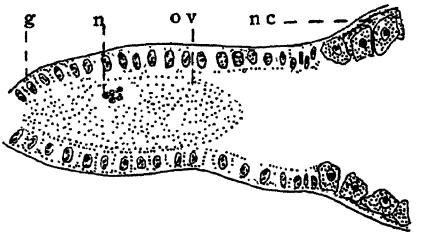

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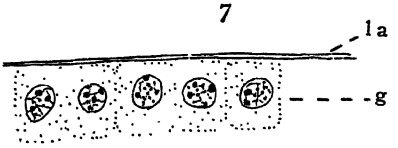

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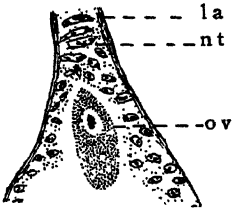

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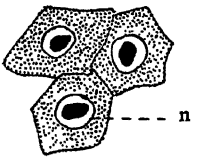

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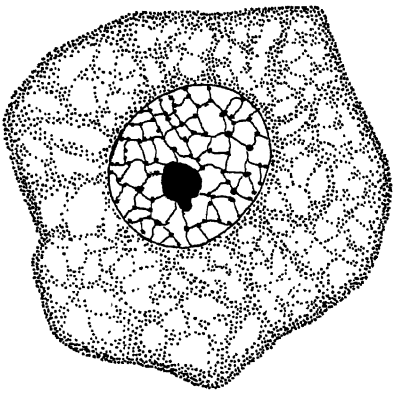

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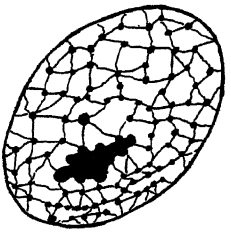

PLATE 4

EXPLANATION OF FIGURES

12 Germarium. × 760.

13 Oocyte in lower portion of germarium near follicular epithelium of vitellarium, cross-section. × 760.

14 Longitudinal section of mature ovum in vitellarium. × 760.

15 Longitudinal section of egg in second cleavage stage with nurse cell body following. × 440.

16 Longitudinal section of egg in blastoderm stage with attendant nurse cell body. × 440.

ABBREVIATIONS

bl, blastomere
cn, cleavage nucleus
con, constricted portion of tubule
ct, epithelial tissue
cy, cytoplasm
g, germ cell
l, lumen
la, lamella of terminal filament
n, nucleus
nc, nurse cell
nco, nutrient cord
nm, nutrient material
nt, transverse cells
o, maternal genital duct
ov, ovum or oocyte
pg, primordial germ cell
pi, periplasm
tf, terminal filament

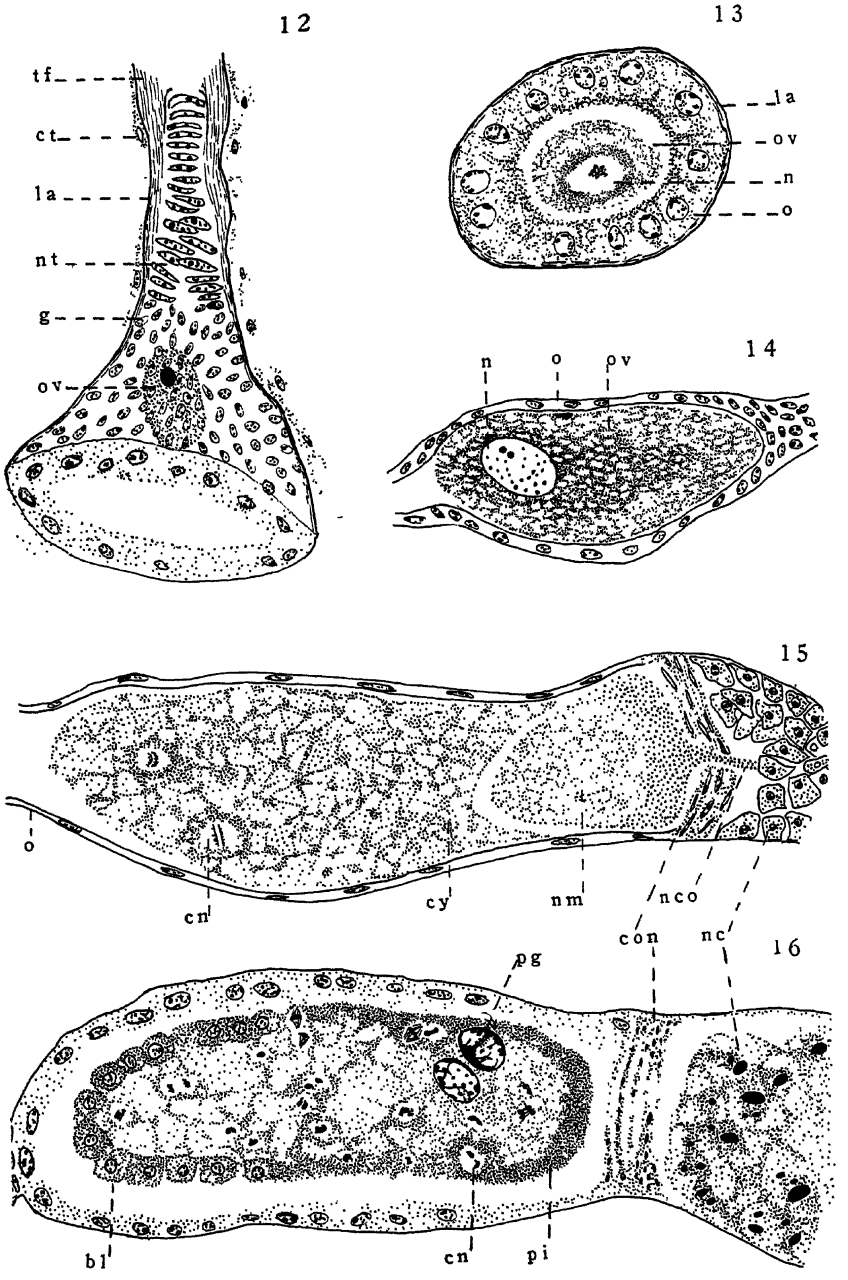
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PLATE 5

EXPLANATION OF FIGURES

17 Cross-section of anterior end of egg in blastoderm stage. × 760.

18 Same as figure 17, about center of egg. × 760.

19 Same as figure 17, in posterior third of egg. × 440.

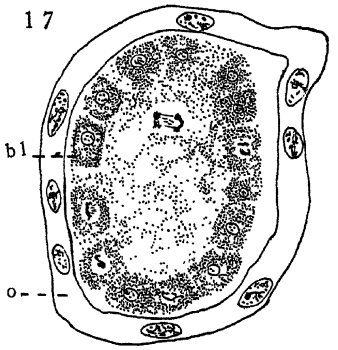
20 Longitudinal section of thoracic and abdominal regions of germ band during segmentation. × 170.

21 Longitudinal section of germ band with segmentation practically complete. × 170.

22 Cross-section of embryo, in region of telson, prior to revolution. × 440.

ABBREVIATIONS

am, amnion
bl, blastomere
ce, cephalic region
cn, cleavage nucleus
fg, fat-globule
m, mesoderm
o, maternal genital duct
p, pleuropodium
pg, primordial germ cell
pi, periplasm
pr, proctodeum
se, serosa
t, trophocyte
te, telson
t1a, prothoracic appendage
t2a, mesothoracic appendage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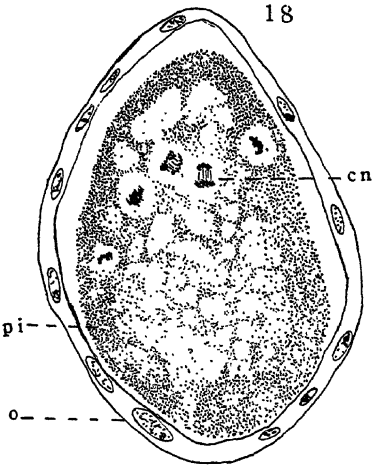
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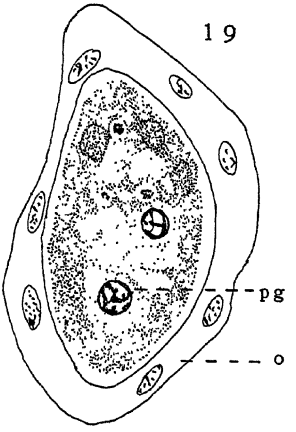
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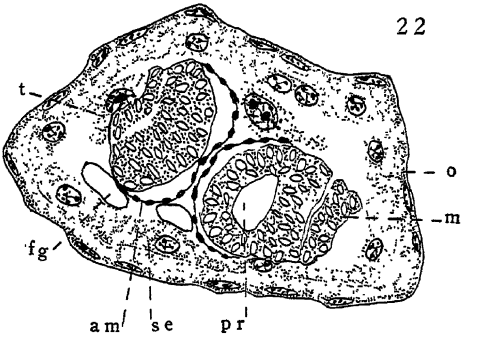
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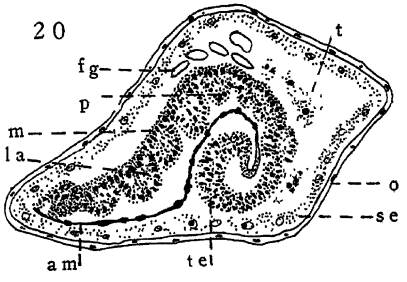
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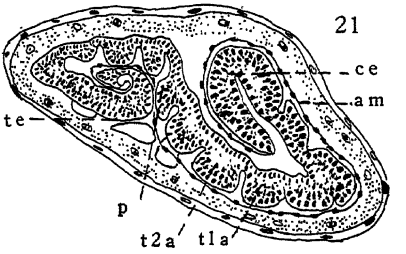
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PLATE 6

EXPLANATION OF FIGURES

23 Cross-section of early embryo through prothoracic region. The space between the serosa and the walls of the oviduct has been slightly emphasized in order to differentiate these tissues. × 440.

24 Same as figure 23 through metathoracic region. See explanation under figure 23. × 440.

25 Same as figure 23, through first abdominal segment. × 440.

26 Section of wall of maternal genital duct and the serosa from *w*, figure 24. See explanation under figure 23. × 1600.

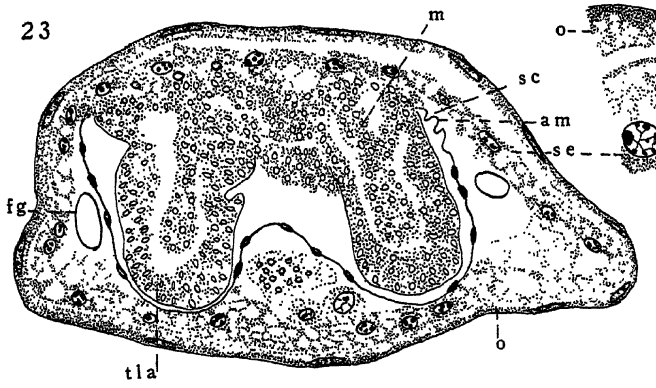
27 Nuclei of amnion from figure 23, enlarged. Free-hand drawing.

28 Nuclei from serosa, figure 25. × 1660.

29 Nucleus of ectoderm cell, postrevolution stage, greatly enlarged. Free-hand drawing.

ABBREVIATIONS

am, amnion
fg, fat-globule
m, mesoderm
n, nucleus
o, maternal genital duct
p, pleuropodium
sc, serosal cavity
se, serosa
t, trophocytes
t1a, prothoracic appendage
t3a, metathoracic appendage
te, telson
v, vacuole
w, section enlarged in figure 26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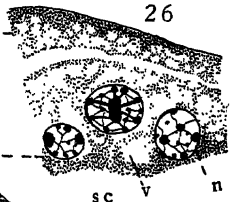
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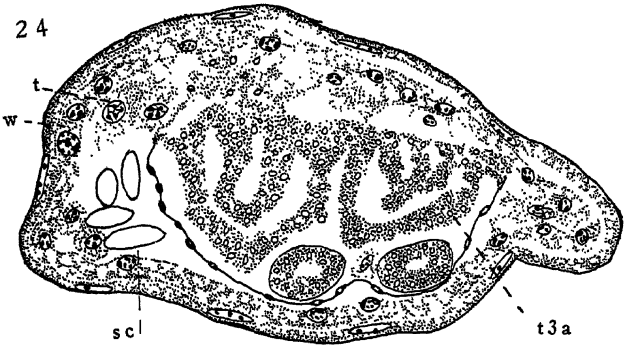
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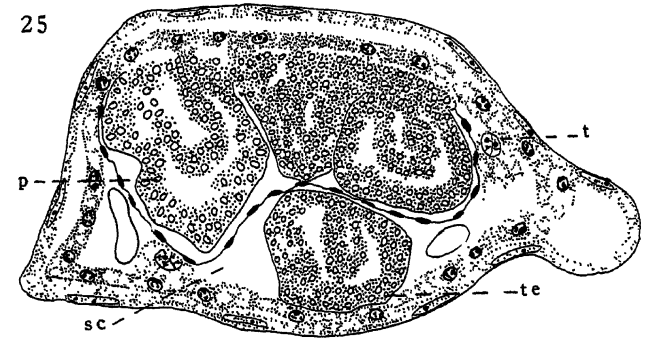
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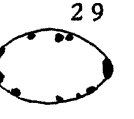
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PLATE 7

EXPLANATION OF FIGURES

30 Cross-section of embryo just before blastokinesis. × 300.

31 Cross-section of embryo after blastokinesis. × 300.

32 Section of pleuropodial extension, greatly enlarged; from *x*, figure 30. Free-hand drawing.

33 Section of pleuropodial extension, greatly enlarged; from *y*, figure 30. Free-hand drawing.

ABBREVIATIONS

am, amnion
ant, antenna
c, central nervous system
fen, formative endoderm
h, hypoderm, ectoderm
hc, haemocoel
m, mesoderm
me, mesenteron
mp, mouth parts
o, maternal genital duct
oe, oenocytes
p, pleuropodium
pc, pleuropodial cavity
pe, pleuropodial extension
se, serosa
t, trophocytes
t1a, prothoracic leg
t2a, mesothoracic leg
t3a, basal portion of metathoracic leg
v, vacuole
x, section enlarged in figure 32
y, section enlarged in figure 33

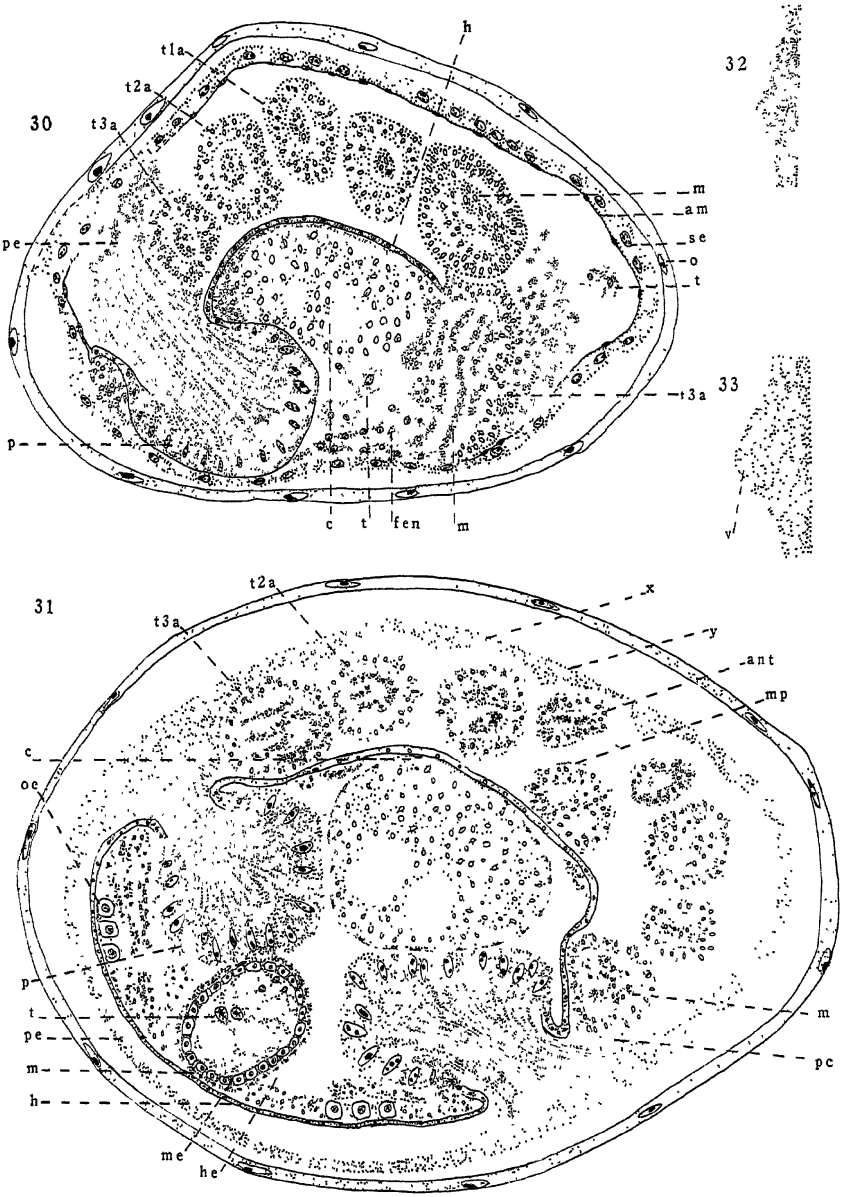
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PLATE 8

EXPLANATION OF FIGURES

34 Postrevolution embryo, prior to closure of dorsal body walls. The pleuropodial extensions are omitted. × 170.

35 Embryonic envelopes anterior to the cephalic region of the embryo shown in figure 33. × 170.

36 Postrevolution embryo a little more than half-grown. Pleuropodial extensions and walls of maternal reproductive tract omitted. Cuticula of appendages shown. × 170.

37 Mesenteron cells, postrevolution stage before closure of dorsal body walls. × 1600.

ABBREVIATIONS

a, appendages
ab1, first abdominal segment
c, central nervous system
c1, supra-oesophageal ganglion
c2, suboesophageal ganglion
c3, thoracic ganglion
c4, abdominal ganglion
c9, ninth abdominal ganglion
ce, cephalic region
cu, cuticula
d, dorsal organ
ee, embryonic envelopes
f, fat-body cells
fen, formative endoderm?
fg, fat-globule
fi, oesophagus
h, hypoderm, ectoderm
hi, hind intestine
l, lumen
m, mesoderm
me, mesenteron
mt, mesothorax
o, maternal genital duct
p, pleuropodium
pr, proctodeum
sm, stomodeum
t, trophocyte
v, vacuole

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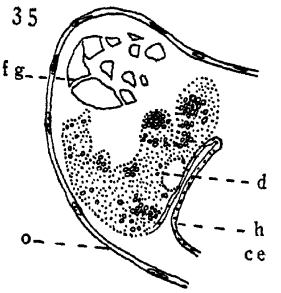
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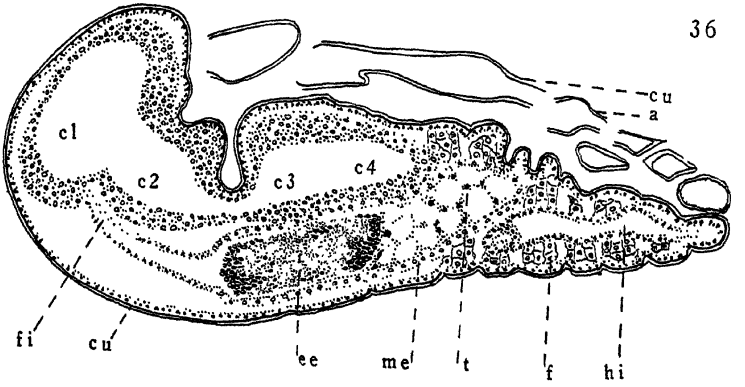
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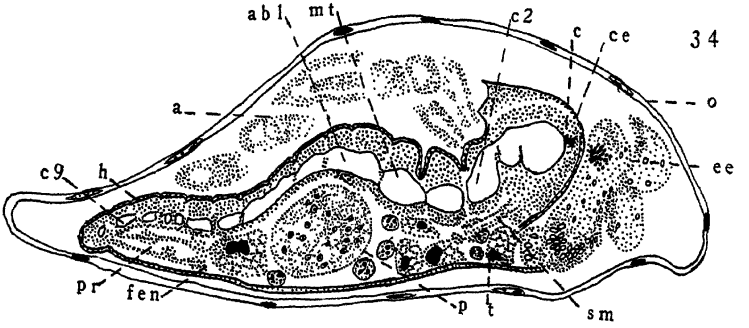
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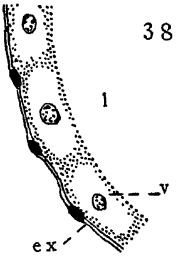
PLATE 9

EXPLANATION OF FIGURES

38 Mesenteron cells after closure of dorsal body walls. × 1600.
39 Mesenteron cells of half-grown embryo. × 1600.
40 Mesenteron cells of nymph, pre-adult stadium. × 1600.
41 Formative muscle fiber, postrevolution stage. × 1600.
42 Muscle fiber, half-grown embryo, nuclei superficial. × 1600.
43 Muscle fiber, precuticular stage, partly striated. × 1600.
44 Muscle fiber, adult female. × 1600.

ABBREVIATIONS

cy, cytoplasm
ex, external epithelium
l, lumen
n, nucleus
r, replacing cell
st, striated portion of muscle
sv, secretory vacuole
v, vacuole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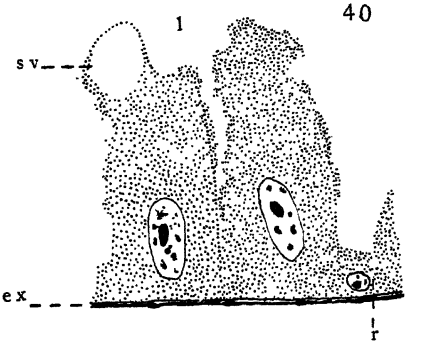
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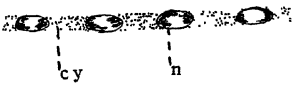
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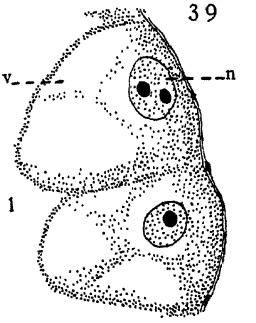
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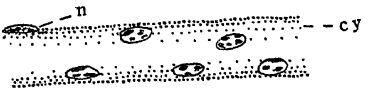
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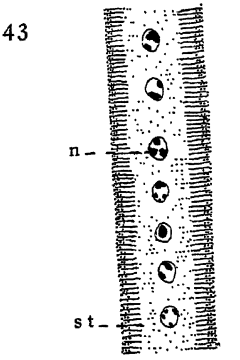
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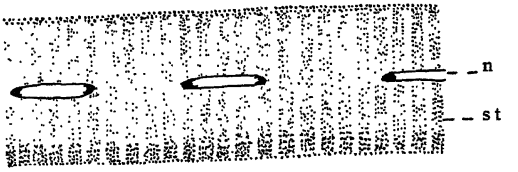
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PLATE 10

EXPLANATION OF FIGURES

45 Cross-section of three muscle fibers from leg of chitinized embryo, showing central location of nuclei. × 1600.

46 Fat-body cell. × 1600.

47 Posterior extensions of paired oviducts (mesodermal) on the point of fusion with common oviduct (ectodermal). × 1000.

48 Oblique section of gonad of chitinized embryo in longitudinal section, showing anterior end not sectioned. × 760.

ABBREVIATIONS

cov, common oviduct
e, epithelium
n, nucleus
pg, primordial germ cell
pov, paired oviduct
v, vacuole

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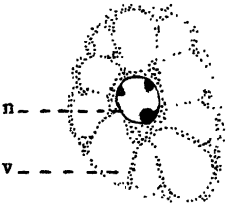

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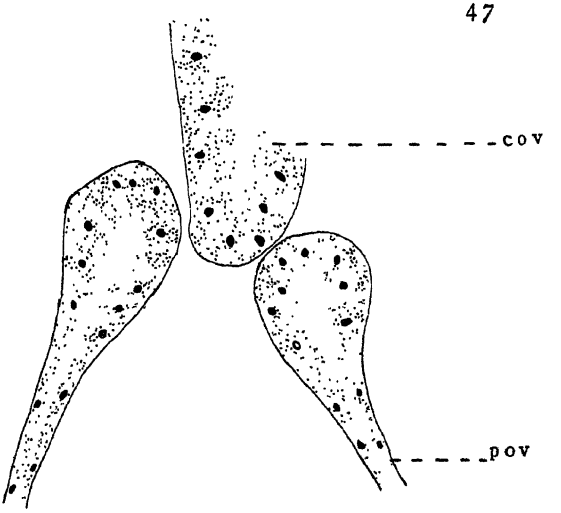

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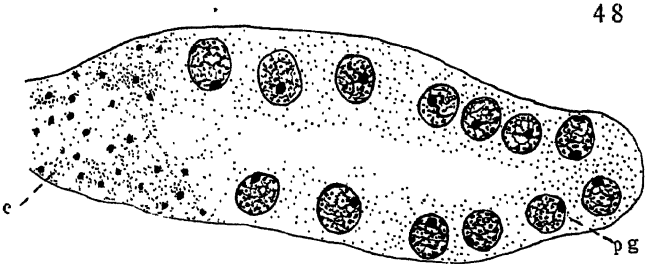

PLATE 11

EXPLANATION OF FIGURES

49 Sketch of whole mount of mesothorax, metathorax, and abdomen of gravid female. × 75.

50 Section of whole mount of female, showing part of pleuropodium of chitinized embryo almost ready to hatch. × 75.

ABBREVIATIONS

ab1, first abdominal segment
ab2, second abdominal segment
ant, antenna
em, embryo
emo, chitinized embryo
fm, fecal matter
mp, mouth parts
mtc, metacoxal cavity
o, maternal genital duct
p, pleuropodium
pe, pleuropodial extension
t3a, metathoracic leg

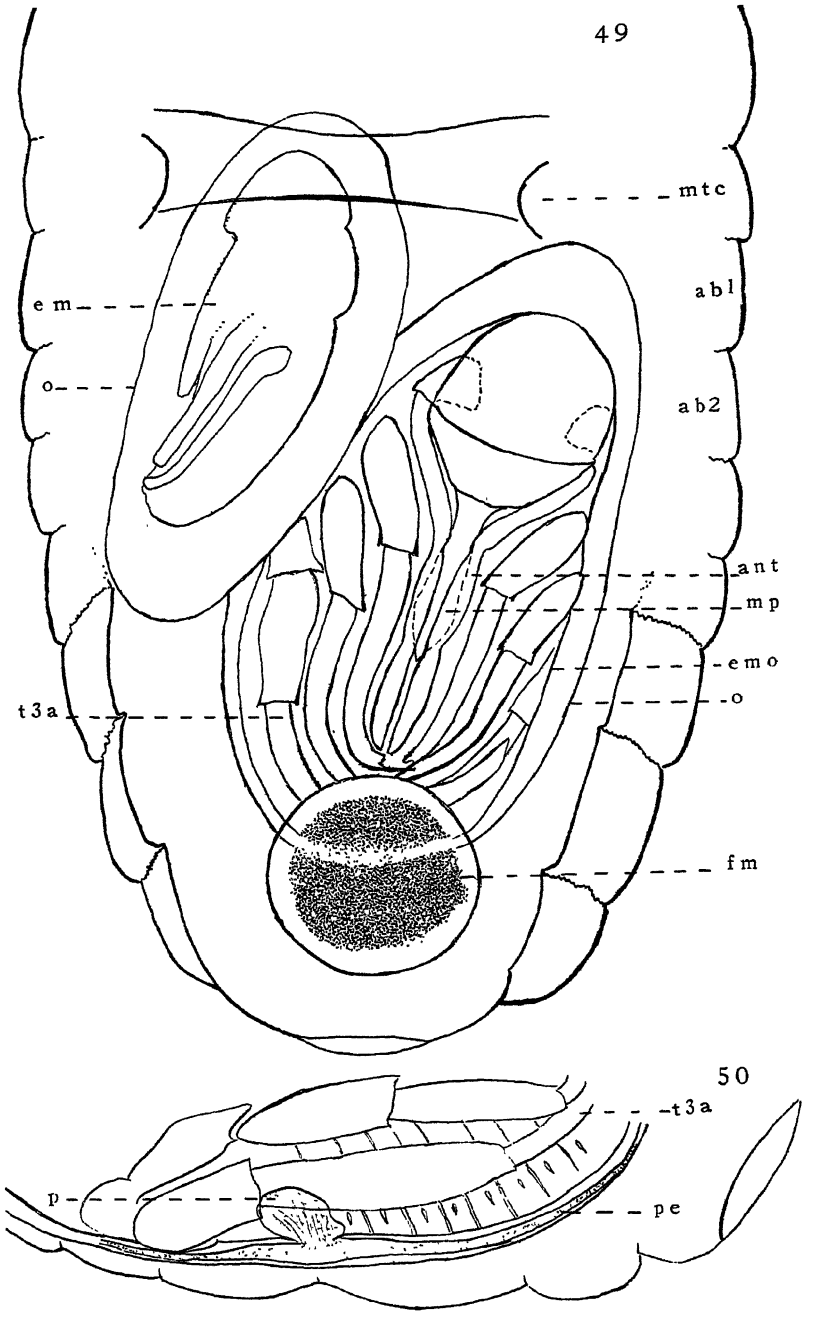
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PLATE 12

EXPLANATION OF FIGURES

51 Cross-section of follicular epithelium between germinal epithelium and the vitellarium. × 760.

52 Degenerating nurse cell body. × 440.

53 Nucleus of amnion beginning disintegration, greatly enlarged. Free-hand drawing.

54 Trophocytes. × 760.

55 Tangential section of pleuropodium showing extension of the cell tips, composite drawing. × 440.

56 Invaginated portion of pleuropodium, chitinized embryo. × 440.

57 Pleuropodial extension at posterior end of chitinized embryo. × 760.

58 Invaginated portion of pleuropodium, chitinized embryo ready for birth. × 440.

ABBREVIATIONS

ch, chromatic material
cu, cuticula
cy, cytoplasm
dgn, disintegrating nucleus
dgp, disintegrating pleuropodium
h, hypoderm, ectoderm
he, haemocoel
l, lumen
n, nucleus
nm, nutrient material
o, maternal genital duct
pc, pleuropodial cavity
pcu, cuticular secretion of pleuropodia
pe, pleuropodial extension
v, vacuole

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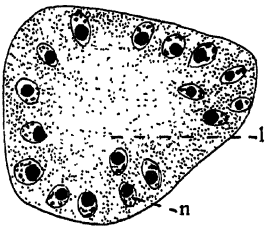
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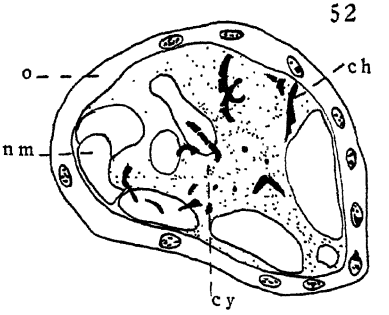
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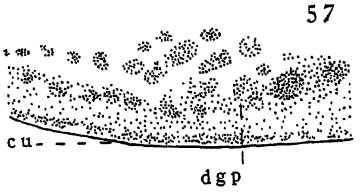
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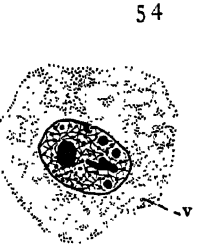
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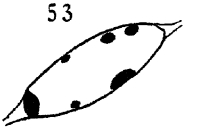
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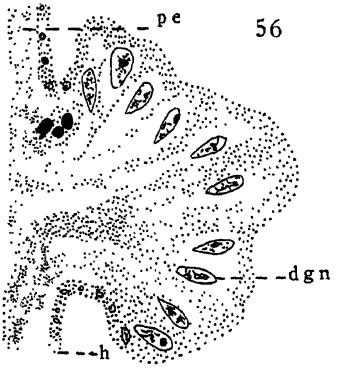
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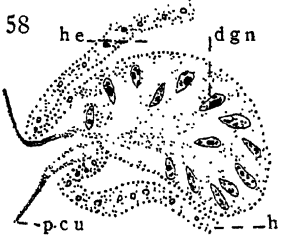
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CHROMOSOME STUDIES

II. SYNAPSIS IN THE TETTIGIDAE, WITH SPECIAL REFERENCE TO THE PRESYNAPSIS SPLIT[1]

W. R. B. ROBERTSON

SIX PLATES (SEVENTY-TWO FIGURES)

AUTHOR'S ABSTRACT

Homologous chromosomes entering parasynapsis are already split. This split evidently originates in each chromosome in the resting period prior to the last spermatogonial division. In the prophase of this division the daughter chromonemata in each half-chromosome were probably incompletely separated, but the succeeding telophase effects the completion of their separation. These processes are accompanied and suceeded by an elongation, straightening, and general paralleling of the split threads and by an elongation of the cell and its nuclear space.

Parasynapsis begins with intimate approximation of the daughter threads in each homologue and is continued by approximation of the homologues at the usual 'bouquet' and 'zygotene' periods. There results the 'diplotene' thread, which therefore consists of four completely distinct strands, but which exhibits a 'two-strand' appearance due to parasynapsis between daughter chromonemata being further advanced than that between homologues.

The pairing process begins at the distal ends and proceeds proximally. With parasynapsis complete, the nucleus enters the 'diffuse' stage. On emergence, disjunction is seen to have progressed from the distal ends to near the proximal ends in each pair and the 'tetrads' have taken form. In the prophase that follows each strand in a tetrad may 'crimp' independently, and with the condensation and matrix(?) formation that accompany it there results the spermatocyte 'tetrad.'

Synapsis is one of the most difficult problems with which the cytologist has had to deal. Its solution is intimately connected with many of the larger problems of biology and any evidence bearing on the subject continues to be of particular interest.

The following account, because of material at hand, has had to be pieced together from cells of six species belonging to three different genera. The story has two gaps, that occurring between figures 25 and 28 and a more serious one between figures 41 and 46. The latter is represented in part by figures 42 to 45. In spite of these breaks, however, in view of the importance that the grouse locusts have assumed as

[1] Contribution no. 121 from the Department of Zoölogy, Kansas Agricultural Experiment Station, Manhattan.

genetic material through the efforts of Nabours[2] and his associates, it seems best to publish the study as it is. Some of the conclusions have already been reported (Robertson, '16, p. 258; '17, pp. 280–281, and '19).

The results here are confined to the male, but in so far as synapsis goes, what observations have been made on the female indicate that conditions there are much similar.

THE PRELIMINARY DIVISIONS

The spermatogonia here included would be classified, according to Davis ('08), as primary (figs. 1 to 5) and secondary (figs. 6 to 24). The former are associated with the apical cell. The latter are in a cyst by themselves and are all of the same stage, occurring in numbers approximating 32, 64, 128, and 256, which would indicate in the case of each group descent from a single primary spermatogonium.

No independent cysts with 16 or lower multiples were found. Figure 1 was from a group of 16 surrounding an apical cell, all alike and but recently divided. Figures 2 to 4 are from a second group of 16 that were associated with an apical cell; 5, from a group of 32 that occurred with this cell, but 6 was from a group of 32 in which no apical cell was present. The relation between spermatogonia in multiples of 8, 16, and 32 which occur with the apical cell and those of 32, 64, 128, etc., that occur free of it is not clear. It might be suggested, however, that the group of spermatogonia leaves this cell behind by the time the 32-cell stage is reached. In the meantime a succeeding younger group may be taking its place.

The chromatin in the late telophase nucleus of an early spermatogonium (fig. 1) is arranged in separate, somewhat zigzag-running threads. These give the appearance of having arisen each from a daughter chromosome by the opening out or loosening up of a much crimpled thread. There is a

[2] I am very much indebted to Dr. R. K. Nabours and Dean L. E. Call, Kansas Agricultural Experiment Station, for the financial assistance which has enabled me to complete this work.

suggestion also that the zigzag appearance might be in part due to there being two chromonemata to each chromosome, such as has been shown for plants (Sharp, '29, figs. 10 and 11).

In the late prophase (figs. 2 to 4) each of the chromosomes is split and usually shows at one end a condensation on each daughter strand. These are probably the polar granules (Pinney, '08). The metaphase chromosomes (figs. 5, 6) show nothing unusual except the great difference in length between the four largest, the *5's* and *6's* and the members of the remaining series. This disparity in lengths comes out especially well in anaphases (fig. 9).

A telophase following the metaphase of figure 6 is seen at figure 7, which was from a cyst containing sixty-three such cells, alike in size and connected, two by two, as shown at the left. The streaming of the cytoplasmic network toward and into this projection is the remains of the old spindle and the projection may therefore be considered the proximal region of the cell. The chromosomes form irregular flocculent-like masses, in the neighborhood of thirteen. The nuclear membrane is not yet established. A nucleolus (*n*) is present in each cell, usually in the periphery of the nuclear region.

Figures 8 and 9 are metaphase and anaphase of secondary spermatogonia from a cyst of sixty-four cells. Portions of four chromosomes, including two long, from the lower daughter group are shown at the bottom of figure 9. It may be noticed that the long chromosomes of the upper daughter group project into the region of the cell which will be constricted later at division of the cytosome, similar to what appears at figure 7. This projection of the long chromosomes may be of significance later in a study of synapsis (fig. 26).

A secondary spermatogonium of the next generation (128-cell cyst) is shown in figure 10. The cells were alike and each contained a nucleolus. In a 128-cell cyst the cells, strictly speaking, should be considered the last generation of spermatogonia, since their division results in what develop into the primary spermatocytes.

THE PRESYNAPSIS STAGES

With figures 11 to 14 begins the transformation from spermatogonia to primary spermatocytes. These telophases and those of figures 15 to 24 follow the last division and are from cysts each containing approximately 256 cells of the same stage. This is also true of all cells shown from figure 15 on.

In figures 11 and 12 are four cells from Tettigidea parvipennis. It is difficult to decide where these should fit into the series from Acridium, 13 to 24, but it seems that they are near the stage, possibly a little earlier, of those of 13 and 14. The chromosomes (fig. 12) are enclosed at their distal ends (in the proximal region of the cell, *p*), each in its own vesicle. At their proximal ends the vesicles have fused and the vesicle walls have disappeared from between the chromosomes. The accessory (*x*) is still isolated in its own vesicle. The nuclei in 12 are stained with hematoxylin; that in 11, with Flemming's tricolor. The chromonemata in 11 resemble those of figure 1 and here also the zigzag appearance may possibly be due to the opening out of the halves of a split chromosome.

In the telophases 13 and 14, from Acridium obscurus, the walls of the vesicles are still intact, at least in a part of the nucleus, and no general nuclear wall has been established. At 14 is a cross-sectional view showing not less than twelve chromosomes. Figure 13 is a lateral view from the same cyst in which variation in lengths may be seen. No split was visible in the chromosomes of either cell, but the stain was heavy, and the threads in each may be compactly crimpled. Attention should be called to the small size of these cells compared to that of figures 10, 7, 5, and 1 from 128-, 64-, 32-, and 16-cell cysts.

Figures 15 to 23 are telophases from the same individual of Acridium granulatus that furnished figures 1, 7, and 10. These cells were stained with Flemming's tricolor. In 15 there are ten separate chromosome bodies, but two seem made up, one of two and the other of three separate parts. Most of the smaller chromosomes appear to be split and are

evidently in the 'opening out' stage. The larger chromosomes appear twisted in such a way that each split half winds about its fellow in a somewhat zigzag manner. Short chromosomes do not show the twisting, the daughter halves lying more nearly parallel. No synapsis has as yet taken place. The nucleolus is in the periphery of the nuclear region and, as above, no typical nuclear wall has yet appeared.

The split in telophase chromosomes following the last division is clearly shown in figures 16 to 21, all from a single cyst, and 22, 23, similar cells from a neighboring follicle. The nuclei in 16 to 21 are slightly later than figure 15, since the nuclear space is more rounded and that between chromosomes is clearer. The cells (19 to 21) are larger than in 15. In a lateral view the chromosomes (16 to 21) have the appearance of a crimpled ribbon, split longitudinally. In cross-sectional view (21) thirteen chromosomes may be seen, all split. Figures 22 and 23 are somewhat later stages from a neighboring follicle. End views of thirteen split chromosomes appear in 23. Nine of the thirteen in 22 show the split. Figure 24 is a cross-section of a much-elongated cell and nucleus, from a second individual, stained with iron hematoxylin. Of the thirteen chromosomes, ten show the split, three not so clearly. There is also some torsion of the split threads here.

In none of the nuclei so far has any side-by-side pairing taken place. In a considerably older cyst from the same testis as 24 are approximately 256 cells similar to figure 25. Above at *a* and *b* are the upper ends of chromosomes marked *a* and *b* in the main figure. Below are shown, from the third section, the four ends, continuations of *a* to *d*, and a nucleolus (?). This cell has increased in size over that of figure 24. The thirteen distinctly separated chromosomes are elongated and have much more space for freedom of movement. All are oriented in the same general direction, possibly in preparation for side-by-side pairing. Staining, iron hematoxylin, is very intense. One chromosome (*x*?) appears smoother in outline and slightly more (?) dense. Just above the cyst

containing this cell, nearer the blind end of the follicle, is a cyst of younger cells, the nuclei of which have chromosomes in the split condition. Figure 24 is one of these cells. In 25 no split is shown, yet the spiral form in some threads indicates its presence and some chromosomes in neighboring cells of the same cyst do show the split. There seems to be a coating or secretion of more intensely chromatic material over these threads which may have covered the split. Also it seems possible, as will be discussed later, that parasynapsis may actually be beginning here, but between the daughter threads of each chromonema. All the chromosomes are pointed at one end, blunt at the other. What appears to be the proximal portion of the cell projects to the right. The fine ends of the chromosomes point in this direction, and these may therefore be the distal ends (opposite to that from which the traction fibers spring) elongating in preparation (?) for the synapsis of figures 30 to 38.

PARASYNAPSIS

The transition from the conditions in 25 to the pairing stages of 27 to 32 or 33 to 38 is represented by figure 26 (?) and possibly by 25a. No evidence of a split is present in the long slender threads of 26 and very little in 25a, nor does there seem to be synapsis in the usual sense going on. The figures, in each case, are of sister cells from the same individual that gave 13 and 14. The sister cells continue to be attached at the region of last constriction of the mother cell. Into this region (in 26) project three long chromosomes whose positions suggest that they have corresponding members in each daughter cell. The cells are much elongated, the right extending downward perpendicularly, the left diagonally. In 25a the sister cells are folded on each other in the direction of the observer.

It might be suggested that this tendency for daughter cells to hang together in the telophase and immediately succeeding stages of spermatogonia concurrently with the prolongation of the telophase condition may be a factor in facilitating the

elongation of these cells and their nuclear spaces at this time. The elongation might permit the chromosomes to get into side-by-side position more easily, as shown at figures 27 and 28. In both the latter there are more than seven threads visible and it may be surmised that maneuvering is taking place for the bringing together of like members.

Accompanying the elongated state is usually a constriction such as may be seen in figures 27 to 32. The significance of this condition is not apparent. The general appearance of the cell at this time resembles that of a sheaf of wheat, and for that reason the cell as a whole may be referred to as in the 'sheaf' stage, although the nucleus is evidently approaching the 'bouquet' stage (figs. 29 to 32 and 33 to 38). The chromatin in the sheaf stage resembles somewhat the first synezesis of other authors (see 'f^1' stage of Wilson, '25, p. 537).

At *qq* in 26 are cytoplasmic bodies which are evidently located in the proximal regions of the sister cells. The same structure occurs in a corresponding position in figures 29 to 32, 33, and 35. There is some doubt about the identity of that in figure 28. The body has some of the characteristics of the idiozome of the bouquet stage (Wilson, '25, p. 329), but its relation to the centriole and Golgi bodies has not been determined. Possibly the latter have been dissolved out by the acetic acid of the fixative (Flemming). It has not been traced into the bouquet stage (36 to 38) in this material. For the present it may be referred to as the *q* body. Its chief use now is in identifying the proximal region of the cells (and distal ends of the chromosomes) during the 'sheaf' stage.

Figures 27 to 32 are from the same individual of Acridium granulatus that furnished figures 15 to 23. The great increase in size of these cells over those of 15 to 23 is noticeable. There is no definite nuclear wall and the elongation continues to give plenty of room for threads to move about. No pairing has taken place in 28. It may be going on in 29.

Figures 30 and 31 are 'sheaf' stages from the same cyst. In 30 the cell is bent partly upon itself and seen here in the plane of the bend—with the bent ends turned away from the observer. The sex chromosome is probably that located at the upper left, although the—in part—dense single chromosome at the lower right has some of its characteristics. The number of autosomes is above six, but less than twelve. Approximation of members toward pairing is taking place. At 31 in the region of the constricted portion appear not more than six threads of about the same thickness. Some of these are double. On tracing them out to the larger part of the nucleus, the thicker threads continue on into finer threads which are more numerous than six. The process of pairing is here evidently beginning at the distal ends of the chromosomes, since this is probably the proximal (p) region of the cell. In 32 from a neighboring follicle pairing is going on, but the ends of some of the threads are more condensed and possibly further advanced than in 31.

Paratettix (figs. 23 to 41) is characterized by much smaller cells than is Acridium or Tettigidea. In 33 and 34 is shown a stage intervening between that of 28 and 31 of Acridium, in which may be seen the parallel condition of what are probably like threads with synapsis itself beginning at the distal ends. Figure 34 is an end view of the same with six pairs and the x-chromosome; 35 is a stage somewhat later, possibly, than 30 to 32 of Acridium, and represents the transition from the 'sheaf' to the 'bouquet' condition. It has six threads and the x-chromosome pointing toward the lower pole.

It should be noted in connection with these 'sheaf'-like stages, 26, 28 to 33, and 35, that the ends of the pairing chromosomes have a tendency to point in the general direction of the q body. In case this body lies to one side of the proximal region of the cell, the ends of the double threads bend in its direction. See especially figures 29 to 33.

Following these stages, the nuclear membrane becomes firm and the nucleus takes on a spherical shape. This throws the pairing chromosomes into the typical 'bouquet' arrange-

ment, as may be seen in the nuclei of figures 36 to 38. The chromosomes are shorter and more condensed at the distal ends. The sex chromosome is more definitely outlined and lies among those ends. At the proximal ends of the chromosomes many of the threads are still unpaired.

Figure 39a shows six threads, all of which appear double. These are continued (as indicated by the numerals *1* to *6*) on into the nucleus shown at 39b. One of the longer paired chromosomes in the upper section continues as two separate threads of half the thickness in the lower section (see numerals *4* and *5* in 39a and 39b). The other longer double thread of 39a behaves in the same way (see numeral *6* in 39b). The remaining four autosome threads are paired throughout. The split shown in the doubles or double portions is probably that of the zygotene. Some of the single threads end in club-shaped masses. The large dense mass above in 39b is probably the *x*-chromosome. From its dense mass there continues a fine single thread.

At 40 is a nucleus in a cell near the stage of 39, in which there are six thick threads and the *x*-chromosome. One of the long threads shows the pairing split. In the remaining five it is not clear. The orientation of the threads similar to that in 36 to 39 is gone.

In 41 is a 'diplotene' nucleus about to enter the 'diffuse' or 'confused' stage of Wilson. There are a few elongated threads, double throughout. One of the three dark masses is probably the *x*-chromosome. The cyst containing this cell follows those of the same follicle in which the first synezesis and pairing are taking place. It is at this stage that one might expect to find the longitudinally quadripartite condition of the paired threads, but more work with possibly different technique is needed to show this. In figure 42 synezesis is shown for Tettigidea. The advanced condition of the *x*-chromosome and the size of the nucleus indicate that this is possibly a second contraction stage ('i' of Wilson).

FORMATION OF THE TETRADS

Following figure 41 is the diffuse stage in which the threads open up, become spun out very fine, and lose their staining capacity. When they reappear toward the end of this period, they lie arranged in all directions and no evidence of a double or diplotene condition may be seen (figs. 43 to 45). But, judging from the next stages, the threads here must be double, although not in the same sense that the diplotene thread is double.

Some threads end in dense club-shaped masses (figs. 43, 44). Figures 45 and 46 have two of these masses connected by the same thread, and in 47 and 48 it may be seen that they are the ends of a tetrad. These club-end stages lead to what we find in 49 to 51 (fig. 50 is on pl. 4), shown especially in 49, where five of the six tetrads with the dense *x*-chromosome stand out. (One half of the smallest, no. *1*, is missing). In this figure at *a* and *e* the tetrads have small transverse arms. The same is shown at *c*, except that the knob portion of one arm has been cut away. In 47 and 48 very small transverse arms appear; in 50 and 51 one large tetrad (no. *6*) shows them more clearly. The two chromonemata making up each transverse arm continue on into the longitudinal arms, one to each. This indicates that the transverse arms as well as the longitudinal are split. Other indications of a split may be seen in the knobs and more bulky portions of the longitudinal arms in figures 46 to 51. From this we may suspect that the threads in the late diffuse stage of figures 43 to 45 are likewise split. More work is needed on the diffuse stages to show this.

When the tetrads of Acridium are followed through the late prophases of 52, 68, 69, and 70 to the metaphase of 71, their four-part structure stands out, and unless a transition from short to long arm has occurred, it may be concluded that their long arms are those from which the traction fibers later spring. If so, it appears that when the tetrads took form, probably back in the middle and late diffuse stage, the members of their pairs that had been in parasynapsis at

the time of figures 39 to 41 must have separated, except in the region of the transverse arms. The latter are very short (47, 48, the *3's* and *6's* in 49, *1's* in 68, *5's* and *6's* in 69) or absent in Acridium.

What has been said of Acridium holds for tetrads, so far examined, in Apotettix (no figures shown) and Paratettix (only one figure, 72, shown). This fact may have led Harman ('15, '20) to conclude that the pairing of the chromosomes in Paratettix was of the telosynaptic type. The earlier parasynapsis stages which are difficult to catch may not have been present in the Harman material at the particular period in which the testis was taken.

When we come to Tettigidea, however, which belongs to a different tribe, the transverse arms may be longer, and cross-shaped tetrads are very common. Their structure may be followed through figures 50 (pl. 4), 51, and 53 to 67. The variation in length of the perpendicular (longitudinal?) compared to the horizontal (transverse?) arms may be seen in the no. *6* tetrad through figures 50, 51, 53, 55, 58 to 67. The lengths of the transverse range all the way from complete absence (61, 64) to equality (67) with the perpendicular (longitudinal?) arms. Also it may be seen that tetrads with and without transverse arms may occur simultaneously in the same group (nos. *5* and *6* in 67; see also figs. 55, 59 to 63), and small chromosomes as well as large chromosomes may possess them (65, 66, 49), even when the larger do not have them. Compare the *3's* with the *4's* and *5's* in 49, the *1's, 2's, 3's* with the *6's* in 61, or the *4's* with the *5's* in 63. But it seems true that the larger members, *5's* and *6's,* are more likely to show the cross type of form.

It is very difficult to prove that up until the time the tetrad lands on the first maturation spindle (65 to 67) what are to be the perpendicular arms have not increased in length at the expense of the shorter, horizontal, arms. However, the simultaneous occurrence of all variations of the cross- with the rod-shaped type of tetrad, also in tetrads of near the same size, in early and late prophase nuclei suggests that very

little, if any, of this transition takes place before the beginning of the anaphase. The tetrads of 65 and 66 may, except for their being more condensed, possess pretty much the same form they had on their appearance in the early prophase (figs. 46 to 51) following the diffuse stage. If this be so, we may consider the perpendicular arms that lie parallel with the axis of the spindle in figures 65 to 67 and 70 to 72 to be the same as the longer arms in the earlier prophases of figures 47 to 64 and 68 and 69.

Some authors are of the opinion that the points on the threads where transition takes place from the horizontal to the perpendicular arms is the region at which crossing over occurs or may have occurred. If so, and if the chromosomes here examined are a fair sample for the four genera concerned, it might be expected that the male in Tettigidea should show crossing-over phenomena more frequently than in Acridium, Apotettix, or Paratettix. According to Nabours ('19, '25, '29) and Nabours and Foster ('29), the last three genera exhibit very little, if any, crossing over in the male. Tettigidea has not been bred sufficiently to know. Nabours' results on Acridium agree with and explain the author's findings in this genus (Robertson, '15, '16) where a heteromorphic tetrad in the cells of one male was seen to exhibit uniformly a segregation of its heteromorphic homologues in the first maturation. And also it should be mentioned that in one of the same papers, '16, two figures (132, 134) from Tettigidea show in the first spermatocyte the no. *6* tetrad dividing in such a way as to raise the suspicion that crossing over may have taken place at some previous time. Perhaps the breeding and cytology of Tettigidea in comparison with that of the other three genera may tell us whether or not these 'chiasmas' are actually the points of crossing over.

Attention should be called to the absence in the Tettigidae family of the ring-form tetrads which are so common in other grasshoppers. The nearest approach to this is the horseshoe-shaped no. *6* of figures 53 and 63. The two largest tetrads are made up of homologues sufficiently long to afford

such rings, but the author has yet to see a ring-type tetrad in this material.

DISCUSSION AND SUMMARY

The chief point to be brought out in this study is that chromosomes are split before they enter upon parasynapsis. This fact has been emphasized by the author in previous publications on the Tettigidae, Acrididae, and Locustidae (Robertson, '16, p. 258; '17, pp. 280–281, and '19). The same was discovered by Bridges in Drosophila ('16) from genetic data. McClung ('27, '28), in Mecostethus, is the first to publish a series of figures showing the condition.

"That such presynapsis splitting is possible, we are led to suppose from the reports of Dehorne ('11) and Schneider ('10) in somatic mitoses and Brunelli ('10, '11) in spermatogonial mitoses, who found that the (anaphase and) telophase chromosomes on their way to the poles showed longitudinal splitting" (Robertson, '16). For plants the same conditions in somatic cells have been demonstrated, especially by Kaufmann ('25, '26 a, '26 b) and Sharp ('29).

Synapsis up to the stage of the bouquet (figs. 36 to 38) and including the stages preceding this back to the termination of the anaphase may in a way be looked upon as an extreme extension of the telophase of the last spermatogonial division. No firm nuclear membrane is established (figs. 11 to 35) until the bouquet stage (figs. 36 to 38) is reached.

In the tettigid material the chromosomes following the last spermatogonial anaphase are left in a parallel position. As they open up, the walls of their vesicles (figs. 11 to 14) that touch each other fuse and disappear, leaving the twelve or thirteen threads or chromonemata (sometimes the x-chromosome vesicle is not included) free and more or less parallel in a large open space (figs. 15 to 23). The walls of these vesicles may be the limiting membrane of the matrix described by Sharp ('29). Each chromonema which is split had evidently been in a much convoluted condition (11 and 15 to 20), as if one had taken a slender pliable double thread

—the two strands of which adhere to each other along one side—and shortened it, without decreasing its diameter, by an irregular crimping. This may have occurred during the preceding prophase. As the vesicle walls disappear the double thread seems to contract, thicken, and straighten out (figs. 17 to 20). During this process it may acquire the appearance of a split ribbon. And as the straightening progresses its daughter threads become gradually more widely separated. Compare figure 24 with figures 16 to 21. In the final stages of the straightening the thread elongates again (figs. 21 to 24). It might be suggested that one function of the telophase is to enable the daughter threads of each chromonema to become completely separated and disentangled, the first opportunity since the preceding resting period in which the split probably originated.

Lying in an open space, the twelve or thirteen straightening double threads become still more parallel. The nuclear space, facilitated by the elongation of the sister telophase cells and unhampered by a nuclear membrane (19 to 24, 26 to 35), becomes gradually more elongated. Coincidently with the elongation of the sister cells and their nuclear spaces go the (continued) elongation and the paralleling (at least in their distal regions) of the thirteen (split) threads (figs. 24, 25, 26). The *x*-chromosome (?) makes a slight attempt at this (25, 28). The result may be what is shown at figure 28.

During this process and later there lies in the cytoplasm the *q* body (26, 28 to 35). Whether it is present earlier (15 to 25a) or later (36 to 38) has not been determined. If the cells in figure 26 are to be considered sister cells, this body must lie in the proximal region of these cells, and therefore in the same region of cells 29 to 35. In the latter, at the ends pointing toward this body, the chromonemata, which have gradually become parallel in two's for perhaps a third to half their lengths (fig. 33), begin their more intimate parasynapsis (i.e., between the already split threads). Since the ends where this begins point toward the *q* body and the proximal region of the cell, it is thought that parasynapsis

must be initiated at the distal ends of the chromosomes. By the time that the process with its shortening and condensation has reached perhaps a third to a half the length of the pairs, the nuclear membrane is established and the 'bouquet' stage is on.

The well-rounded 'bouquet' nucleus may be considered the termination of the telophase of the last spermatogonial division. The cell, were it in ordinary mitosis, would be ready, following this, to enter upon the 'resting' period, but the zygotene (39a, 39b) pachytene (40?), and early diplotene (41) stages intervene. The absence of any U-shaped chromosomes in the diploid cells of the Tettigidae is fortunate in enabling us to check up on the loop-like formations that have been described for so many species in the 'bouquet' nucleus. It may be seen here (figs. 36 to 38) that whatever loop-like threads there are present have at one end the pairing in progress, while at the other ends these loops continue into single threads (37, 38). It may be concluded that what loop formation there may be present is the result of accident and of no significance.

The zygotene shows the chromosomes to be still all oriented in one general direction, but this common orientation is lost in the pachytene, if figure 40 is the pachytene, and the diplotene (41). The split showing in the zygotene and pachytene is that resulting from parasynapsis. The question arises what has become of the presynapsis split (figs. 15 to 25). It was definitely present when the cells and their thirteen threads entered on the elongation process (fig. 24 and sister cells to 25). There is some evidence that a torsion (Wilson and Morgan, '20; Robertson, '16, '19) was also present in each of the split threads at this time. The disappearance of the split may be due in part to it having been covered up (fig. 25) and in part to the attenuation that the chromonemata undergo during the sheaf and later stages (figs. 25a to 34). That the split of the pairing threads from 31 to 40 is not the presynapsis split may be concluded from the fact that in the latter thirteen separate threads (15 to 25) are

concerned, with no pairing (except possibly between the halves of a thread), while in the former it is a question of at most six double (paired) threads at one pole of the nucleus leading off to a larger number of unpaired threads of half the thickness at the opposite pole. The sex chromosome, of course, is present.

As suggested in the section on 'The presynapsis stages' the disappearance of the presynapsis split may be in part the result of parasynapsis making its appearance for the first time by bringing together the now widely separated halves (fig. 24) of each split thread, including those in the *x*-chromosome also. The latter differs very little from the other twelve threads up to this point, even when it lies in its own isolated vesicle (if such belongs to it) in figure 12. Figure 24 is from a slightly younger cyst in the same follicle as figure 25. In the former the thinness of the daughter threads and, in the latter, the shortness and condensation of the thirteen, probably split, threads resemble very much the fineness of the single threads and the condensed and shortened condition of their paired portions in figures 30 to 38. Following 25, the thirteen threads elongate and become fine. No evidence of the split may be seen in the autosomal threads from figures 26 to 38. Their fineness, of course, may be a reason, but may it not also be due to parasynapsis? There may be some doubt about 26 being a stage immediately succeeding 25, but 28 probably belongs near the next stage, and there is no evidence of a split in the threads of 28.

The stages between 30 and 40 might be looked upon as a second step in the process of parasynapsis—the coming together in pairs, beginning at the distal ends (figs. 33 and 34), of the twelve split threads. This explains in part the behavior of the *x*-chromosome, whose increased condensation dates probably from 35 on. Its extremely condensed condition (figs. 35 to 42) might be looked upon as due to a double amount of or a doubly long exposure to the synaptic influences. This suggestion might also explain why a condensed condition of the *x*-chromosomes is not found in cases where there are two such present, e.g., in the female.

The author presents this view—that there are two steps in parasynapsis, the bringing together of the split halves of a chromosome first and later each pair of split homologues—with very much caution in connection with this material. He feels that he has made insufficient study of the stages between figures 24 and 28. Whether the suggestion is acceptable or not, it seems that with the establishment of the diplotene (fig. 41) the synaptic processes are near their completion. The split apparent in the diplotene (41) is probably that of synapsis between the homologues. The presynapsis split might also be expected under the best of conditions of material and observation, in which case the diplotene thread should show a quadripartite structure. But the presynapsis split may be hidden, due to the previous synapsis between the halves of each homologue. This might account for the persisting doubleness of the diplotene thread. From the pachytene and diplotene on, the elongation of the threads takes place. The *x*-chromosome makes an attempt at this also (fig. 45), but may be unable to overcome the relatively double dose of synapsis which it has undergone (?).

As to what happens in the diffuse condition may only be conjectured from what has preceded and what follows (43 to 72). Pinney ('08) and Wenrich ('16) showed that the spermatogonial chromosomes in Phrynotettix were polarized, and had polar granules at the ends, from which the traction fibers later arose. In Tettigidae spermatogonial chromosomes show the same polar granules (figs. 2 to 4) and there is no reason to believe that the disjoining portions of a tetrad at maturation (figs. 65 to 67 and 70 to 72), which are—disregarding cross overs (?)—the equivalent of spermatogonial homologues, have changed the points or ends from which their traction fibers spring. If this be correct, the perpendicular (longitudinal) arms of the tettigid tetrad may be considered the portions of the pair that have become separated, in some cases completely (telosynaptic position), in others incompletely (cross type with transverse arms). These longitudinal as well as transverse arms (when they occur)

are split. We have reason to believe that the pair that entered the diffuse stage in parasynapsis (41) reappears (43 to 51) in pretty much the condition of the prophase and metaphase tetrad (figs. 52 to 72).

This process of disjunction and tetrad formation must take place during the diffuse stage. There is further evidence of this in the club-end pairs which appear in the late diffuse stage (43 to 46) and lead to the club-end tetrads of figures 49 to 52. There is some indication of two such pairs present in the zygotene stage (39a, 39b) similar to what Gelei ('21) described for Dendrocoelum.

The presence of tetrads of the cross type in the male of Tettigidea and the comparative absence of this type of tetrad in the males of Acridium, Apotettix, and Paratettix offer in connection with Nabours' breeding work an opportunity to test the hypothesis as to whether or not such chiasmas are connected with the phenomenon of crossing over.

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EXPLANATION OF PLATES

Material

The drawings are by camera lucida and (excepting 24) appear here at a magnification of 3100 diameters. Fixation was by Flemming's fluid. For stain Flemming's tricolor was used for figures 1, 7, 10, 11, 15 to 23, 27 to 32 and Heidenhain's hematoxylin for all others. The material used is from nature and consists of sixteen males (individuals A to P) belonging to six different species and three genera as follows.

Acridium granulatus: A, figures 1, 7, 10, 15 to 23, and 27 to 32; B, figures 24 and 25.

A. obscurus: C, figures 13, 14, 25a, 26, 43 to 46, 71; D, figures 47 to 49, 52; E, figure 69.

A. ornatus: F, figure 68.

A. incurvatus: G, figure 70.

Paratettix texanus: H, figures 33 to 41; I, figure 72.

Tettigidea parvipennis: J, figures 2 to 4, 8, 9, 51, 53, 55, 57 to 60; K, figures 5 and 6; L, figure 11; M, figures 12, 42, 61; N, figures 50, 63, 64; O, figures 54, 56, 62; P, figures 65 to 67.

PLATES 1 TO 6

EXPLANATION OF FIGURES

1 to 5 Primary spermatogonia. 1, nucleolus (*n*) in each; remains of spindle at connection with sister cell to right; *p*, proximal region of cell; 2 to 4, group from single rosette showing polar granules; 5, six pairs of autosomes, *1* to *6*, and sex chromosome, *5x*.

6 to 10 Secondary spermatogonia. 6, from thirty-two-cell cyst; 7, telophase from an (approximately) sixty-four-cell cyst; *p*, proximal region of cell, to left; 8 and 9, metaphase and anaphase; below in 9, four fragments of chromosomes from sister group; 10, telophase.

11 to 23 Telophases of the last spermatogonial division. 11, fragment (Tettigidea) showing two early telophase vesicles with chromonemata; to right, chromonema without vesicle walls: 12, similar stage (Tettigidea) under hematoxylin; sister cells at upper left; *p*, proximal regions of cells; *x*, sex-chromosome vesicle: 13 and 14, oblique and end views (Acridium); interchromosomal vesicle walls disappearing; cells small compared to figure 1: 15 to 23, opening out of crimpled chromonemata (Acridium) and showing presynapsis split, 15, from one and, 16 to 21, from another cyst in same follicle; 16, all the chromosomes shown, from two sections; 17, 18, 19, and 20, fragments of cells and nuclei; 21, nucleus only; 22, 23, from another follicle, same testis.

24 Cross-section of elongated cell (Acridium), later stage of presynapsis split, slightly lower magnification.

25 Lateral view (Acridium) showing elongation of cell and of its thirteen chromosomes; presynapsis split for the most part covered, but some ribbon-like appearance still present; distal ends of chromosomes and proximal region of cell to right.

25a End view of sister cells (Acridium) bent upon each other; stage between 25 and 26.

26 Elongation of sister cells and chromonemata (Acridium) entering 'sheaf' stage and probably synezesis; *p*, proximal regions of cells and attachment; ends of three long chromosomes correspond in each cell; *q*, idiozome-like body.

27 to 32 'Sheaf' stages (Acridium). 27, part of cell, and 28, complete cell, first contraction stage (synezesis); 27, from younger, and 28, from older cyst, same follicle; threads parallel and showing constriction with maneuvering for pairing; 29 to 32, coming out of synezesis with synapsis and zygotene-like condensation beginning at distal ends of chromosomes; *q*, idiozome-like body present in all cells of these cysts; 29, extreme extension of threads; 30 to 32, from same follicle as 28, but next older cyst; 31, upper end of cell rises up to meet observer; 32, pairing and zygotene-like condensation.

33 to 41 'Sheaf,' 'bouquet,' zygotene, pachytene (?), and diplotene stages (Paratettix) from single individual. 33 and 34, lateral and end views of near 'sheaf' stage; like threads parallel and more intimate synapsis beginning at distal ends; 35, transition from 'sheaf' to 'bouquet'; chromosome to right moved out from underneath; 36, 37, 38, 'bouquet' nuclei in rounded form and with nuclear membrane well established; 39a and b, zygotene nucleus, in two sections, from older cyst in same follicle as 33; proximal ends of six threads of a continued on (see numerals) in b; 40, probably pachytene (?) nucleus; six thick, short threads and *x*-chromosome (?); zygotene split apparent in one; parallel orientation gone: 41, diplotene nucleus; threads fine and much elongated and diplotene split clear; parallel orientation gone.

42 Nucleus of second (?) contraction stage from Tettigidea.

43 to 46 Late 'diffuse' stages (Acridium); two threads end in club-shaped masses; *x*-chromosome sausage-shaped; split condition of threads not visible except in club-shaped masses (45, 46).

47 and 48 Early diakinesis (Acridium) showing tetrads with club ends and slight transverse arms and with mostly telosynaptic position of homologues; split is apparent in bulkier portions.

49 Slightly later stage (Acridium); one-half of no. *1* missing; chromatin-like threads streaming off into cytoplasm from ends of no. *5* homologues.

50 (pl. 4) Early diakinesis (Tettigidea); tetrads with mostly telosynaptic position of homologues; no. *6* has short transverse arms; *x* bent to U shape.

51 Slightly later diakinesis (Tettigidea); five tetrads, with homologues in telosynaptic position and showing club-like ends; no. *6* has transverse arms; split apparent in club ends and at chiasma.

52 Middle diakinesis (Acridium); tetrads with homologues in telosynaptic position.

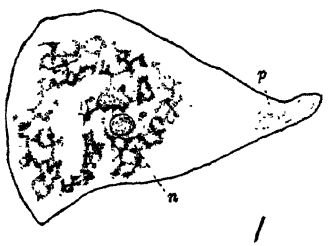
53 to 64 Tetrads in middle diakinesis (Tettigidea) showing four-part structure and gradual shortening and condensation; all tetrads and *x*-chromosome shown in 55 to 64; some with completely telosynaptic position of homologues; others of the cross type with transverse arms of various lengths.

65 to 67 Metaphases, primary spermatocyte (Tettigidea), showing tetrads of telosynaptic and cross type.

68 and 69 Middle diakinesis; 70, late diakinesis (Acridium), showing all tetrads with mainly telosynaptic position of homologues.

71 Metaphase (Acridium). Telosynaptic position of homologues in tetrads.

72 Metaphase showing same in Paratettix.



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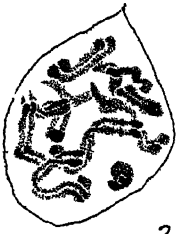
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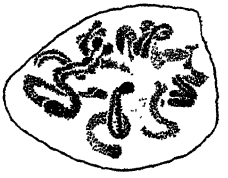
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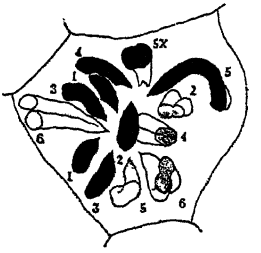

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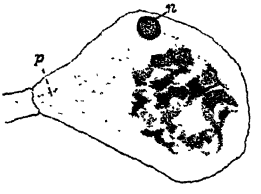

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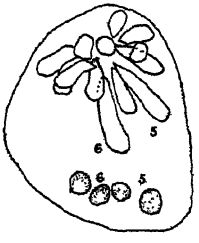
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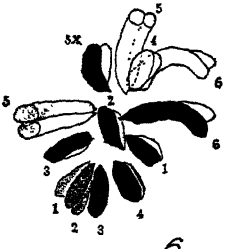

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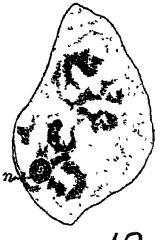

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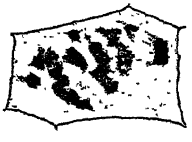
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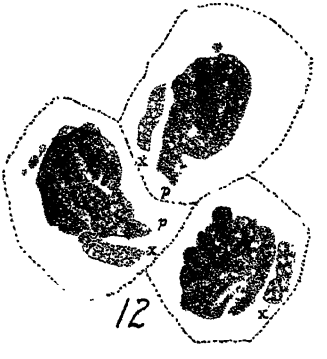

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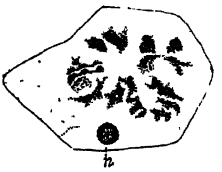

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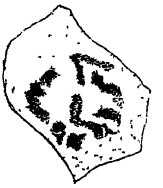

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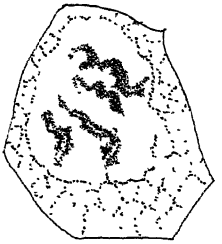

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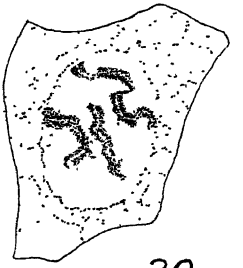

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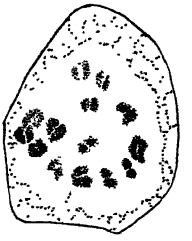

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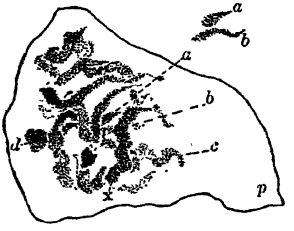

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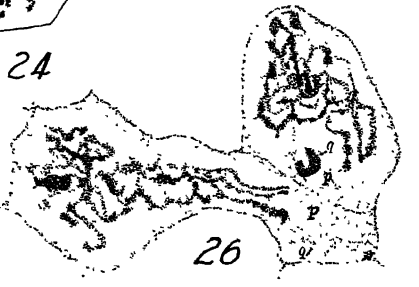

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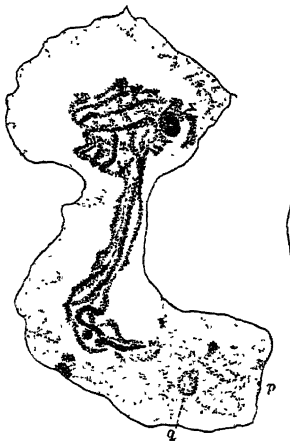

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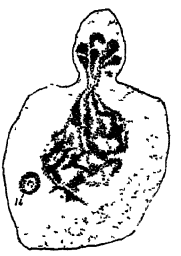

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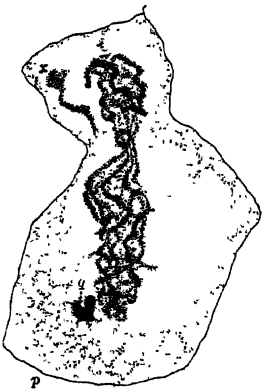

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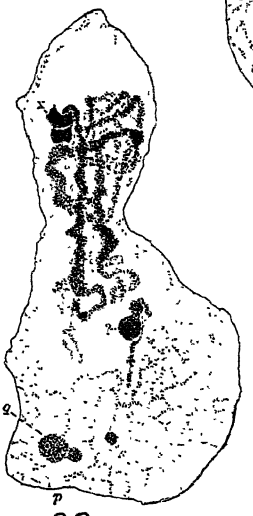

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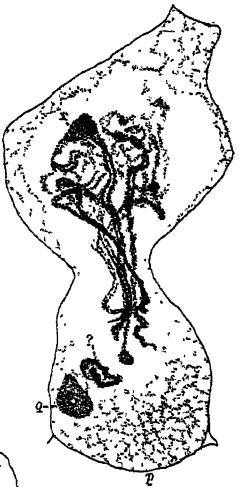

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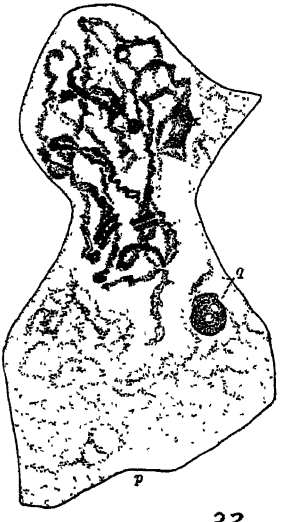

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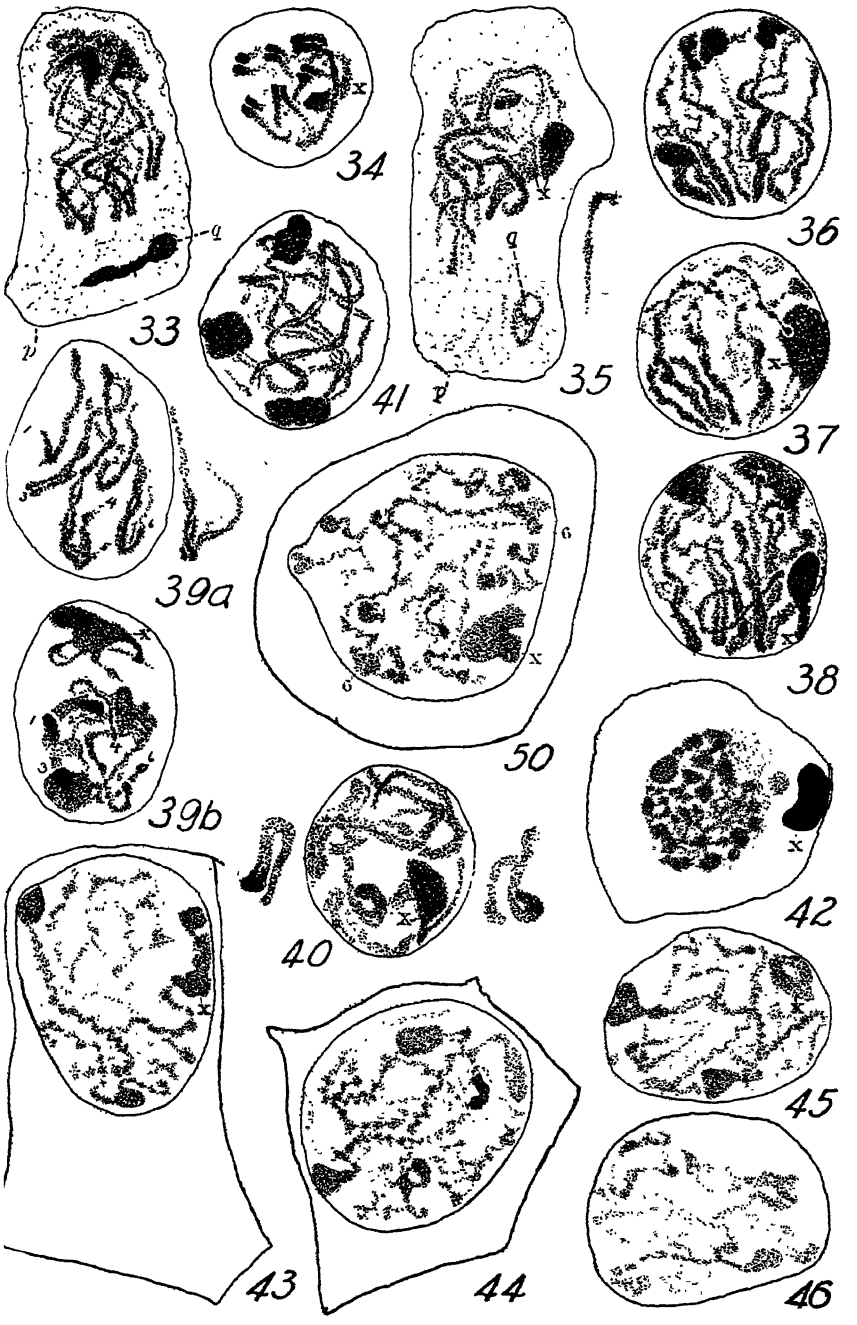
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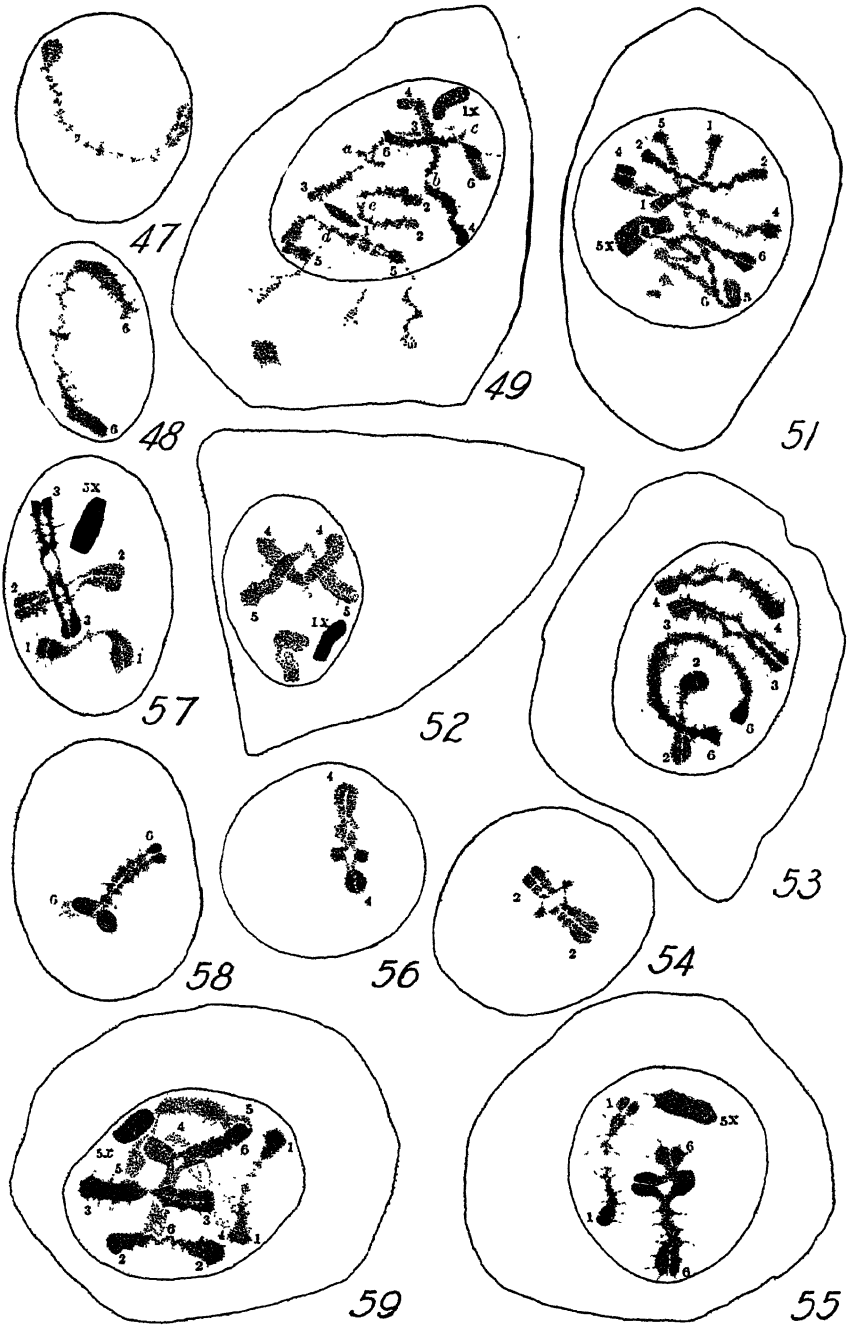
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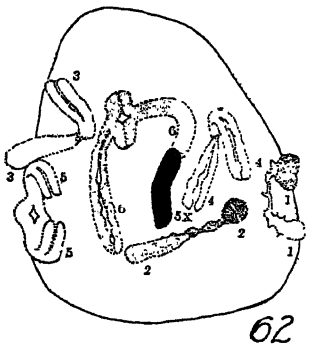

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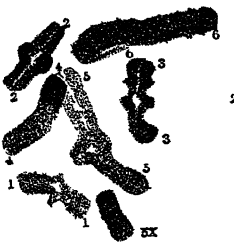

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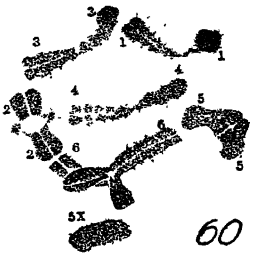

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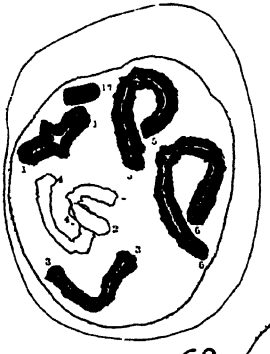

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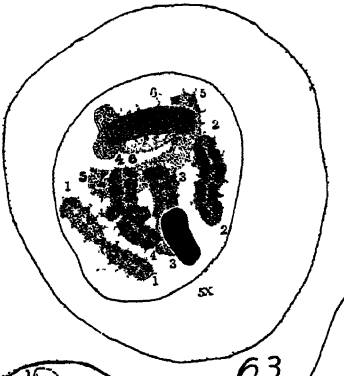

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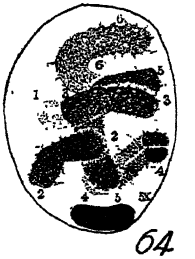

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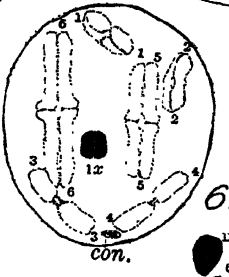

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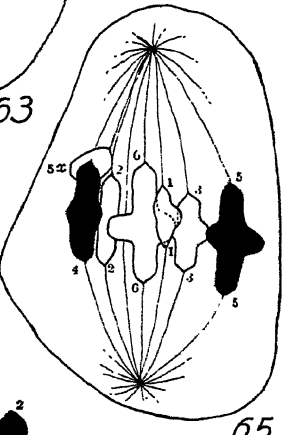

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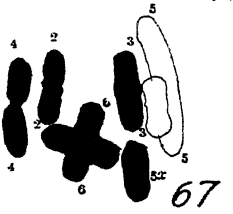

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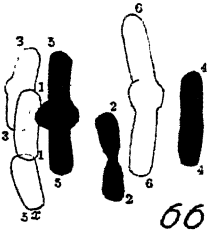

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AN INVESTIGATION OF THE NERVOUS SYSTEM AS A POSSIBLE FACTOR IN THE REGULATION OF CILIARY ACTIVITY OF THE LAMELLIBRANCH GILL

ALFRED M. LUCAS

Washington University

FIVE TEXT FIGURES AND FOUR PLATES (TWENTY-ONE FIGURES)

AUTHOR'S ABSTRACT

Certain variations of ciliary activity in the lamellibranch gill occur which are an intrinsic part of the gill tissue and which are due to causes other than environmental changes. Experimental and morphological evidence indicates that the central nervous system is not involved in the production of these variations.

A comparative study of laterofrontal and lateral ciliated cells leads to the conclusion that the coordination impulse passes through the cytoplasm of the cell and that the velocity of the propagation wave is influenced by the number of cell walls per unit length through which it passes. It is suggested that the ciliary rootlets in the laterofrontal cells, due to their arrangement, bring the impulse simultaneously to both rows of cilia within a single cell.

CONTENTS

Introduction 147
Literature 148
Material and methods 151
Observations 153
General organization and behavior of the cilia of the gills 153
Normal variations in ciliary activity 156
Cytology of ciliated epithelium 161
Occurrence and movement of gill muscles 166
Gross morphology of the nervous system 169
Histology of the nervous system 173
Electrical stimulation of the nervous system 175
Discussion 176
Summary 182
Bibliography 183

INTRODUCTION

Investigations of ciliary movement on various ciliated structures of animals have led to two general ideas as to the locus of the factor controlling the movement. The control of the movement of cilia has been attributed by Parker ('19)

to the ciliated epithelium itself, and not to any portion of the animal outside the cells which bear the cilia. Others (Grave and Schmitt, '25; Carter, '26 and '29; Copeland, '19 and '22, and others) have found evidence which indicates that the nervous system of the animal may exercise some regulatory influence in ciliary activity. Grave and Schmitt ('25) have described a system of intracellular fibers, in a certain group of ciliated cells of the lamellibranch gill, which they suggested to be a mechanism through which such control may be exerted.

The present work involves observations upon the normal behavior of living cilia of lamellibranch mollusc gills, upon the finer structures of the ciliated cells, on the distribution and histology of the nervous system, and, finally, upon the effect of electrical stimulation of the nervous system.

The problem was suggested by Prof. Caswell Grave, and to him I wish to express my thanks and appreciation for his help and direction during the progress of the work. Thanks are due Prof. Joseph Erlanger, who kindly extended the use of equipment in his laboratory for the electrical stimulation experiments; Mr. W. M. Patterson, for his aid in the tabulation of vibration frequencies of ciliary movement; and Dr. Bryant Walker, who identified the fresh-water molluscs used in this study.

LITERATURE

Much of the previous work concerns the movement of a single cilium, its physiology and its relation to other contractile tissues. Some contributions pertain to the coordination of cilia in their movement, and recently several investigators have sought for some organ or mechanism regulating ciliary activity as a whole. It is only with the last subject that this section of the paper is concerned. Some authors consider the ciliated epithelium to be altogether an autonomous organ and that the mechanism both of coordination and regulation of activity is inherent in the epithelium itself.

Such an idea is expressed by Parker ('19) and he has designated this "elemental property of protoplasmic transmission" as that of neuroid transmission. He states (p. 65):

> The fact . . . . that no one has ever been able to control ciliary activity through nerves and all the cases of ciliary coordination thus far brought forward can be explained on the basis of neuroid transmission, renders the belief in the nervous control of cilia very improbable. In fact, it may be stated that at present there is not the least ground for the assumption that true nervous activity is in any direct way involved in ordinary ciliary reactions.

He states further:

> These impulses pass through the deeper protoplasmic parts of the tissue and call forth the successive activity of the cilia which thus gives evidence of this transmission wave.

Grave and Schmitt ('25) are the first to describe a morphological structure along which a coordinating impulse may pass. Agersborg ('23), studying the morphology of the nudibranchiate mollusc, Melibe, describes and pictures a group of ganglion cells and fibers just below the ciliated epithelium which lines the groove on the ventral side of the foot. The fibers extend to the bases of the ciliated cells, but the nature of the termination is not shown. He suggests that the cilia of the ectoderm of the foot are under nervous control.

Carter ('26 and '29), studying the nudibranchiate veliger, traced nerve fibers into the ciliated epithelium of the velum and described their terminations in the ciliated cells. His experimental work, combined with his morphological study, presents the most convincing evidence that the ciliary activity is under the influence of the nervous system.

Other investigators observing the behavior of ciliated epithelia have felt that their observations indicated that some controlling mechanism is present and effective. Babak ('13), studying the factors regulating the movement of water over the gills of lamellibranchs, noted periods of activity and of inactivity of the cilia that led him to suggest the possibility of a nervous control of this movement.

Copeland ('19), studying the movement of cilia on the pedal discs of two species of gastropod, observed that the movement discs of two species of gastropod, observed that the movement of the cilia of the foot was responsible for the movement of the animal, and that the speed of ciliary movement varied from a maximum to complete stoppage. When the foot is cut from the body, its muscular and ciliary activity is irregular for the first twenty-four hours, but after this period the foot remains expanded and the cilia continue to beat at a uniform moderate speed, without stoppage. He concludes that the controlling mechanism is the nervous system, but he was not able to determine whether the impulse is carried to the ciliated epithelium directly or whether to the muscles underlying the epithelium, the muscles, by their expansion and relaxation, producing the impulses that control the ciliary activity. Copeland ('22) repeated this work on another gastropod, Polinices, and by cutting a square in the base of the foot, he was able to demonstrate that the impulse is carried from the subepithelial tissue. Since the cilia within the circumscribed area behaved as those outside, it was concluded that the impulse governing ciliary movement is nervous in origin.

Merton ('23) found that electrical stimulation of nerves leading to the ciliated epithelium of the snail's lip produced activation of previously quiescent cilia.

McDonald, Leisure, and Lenneman ('28) found that electrical and chemical stimulation of sympathetic fibers which innervate the ciliated epithelial lining of the frog's pharynx causes the cilia to beat rapidly, and that stimulation of parasympathetic fibers causes the cilia to beat slowly.

Göthlin ('20), after a study of the swimming plates in Beroë, suggests that the activating impulse arises in the nervous system and is conducted through the nerve net to the swimming plates, but that in the swimming plates the impulse is transmitted from cell to cell.

MATERIAL AND METHODS

The ciliated epithelia upon which observations have chiefly been made were obtained from two marine species, Mytilus edulis and Modiolus modiolus, and from two fresh-water mussels, Amblema costata and Megalonaias gigantea. For the studies of the nervous system M. edulis and A. costata and M. gigantea supplied the material. Permanent preparations of the mantle and gills were made from M. edulis and A. costata. Living gill tissue from each of these species was observed for ciliary activity.

The body of the mollusc was removed from the shell for experimentation, and in this process of removal the adductor muscles were cut.

In the study of living ciliated epithelium, filaments of the gill were removed and placed upon microscopic slides, usually without a cover-glass. The Chambers microdissection apparatus was employed in some procedures.

Studies of ciliary activity while the gills were attached to the mollusc were made in a special container constructed as follows: A Petri dish ¾ inch in depth was nearly filled with a mixture of wax and paraffin, in which, after hardening, a depression was excavated, the size and shape approximating the length and thickness of the mollusc, as viewed from the dorsal side. This depression was located about 1½ inches from one side of the dish. A second smaller depression beside the former was excavated to the bottom of the dish for admitting light. The dorsal side of the mollusc was placed in the larger depression, and the mantle and outer gill of one side were folded back into the same depression. The inner gill of this side was then placed over the smaller opening in the wax and the container placed upon the stage of the microscope. Water was kept flowing over the preparation during the period of observation from an aspirator bottle, placed above the level of the microscope stage, through a rubber tube ending in a pipette drawn out to a small diameter. The point of the glass tube was placed below the surface of the water, in order to prevent any disturbance of the surface.

The overflow was conveyed from the dish by means of one or two short pieces of wool yarn suspended over the side.

To ascertain the effect of electrical stimulation of the nervous system upon ciliary activity, the mollusc was placed in the container just described and the ciliated epithelium of the gills was observed under the microscope while either the branchial nerve or the cerebrovisceral connective was stimulated with an induction current.

Macerated preparations of filaments of the gills of Amblema costata and Megalonaias gigantea were made with numerous macerating fluids, of which 3 per cent chloral hydrate and 3 per cent sodium borate proved to be most satisfactory, and of these sodium borate was finally used exclusively. A trace of iodine or aqueous eosin was frequently added to produce a faint color differentiation.

Pieces of the gill of Amblema costata and of Megalonaias gigantea were placed in the macerating fluid for varying lengths of time, from one-half hour to several hours, then removed, and scrapings from them transferred to microscopic slides.

For permanent preparations, several fixatives were employed, including: Bouin, Allen's B_3, Champy, 10 per cent formol, and saturated corrosive sublimate.

Various staining methods were used. For the finer structures of the ciliated cells Heidenhain's iron haematoxylin with acid fuchsin was found to be most satisfactory. Very good results were also obtained by using Ehrlich-Biondi's triple mixture and Ehrlich's haematoxylin with acid fuchsin. Differentiation of various tissue elements was obtained by the use of Mallory's triple connective-tissue stain, neutral gentian (Bensley, '11), erythrosin, and toluidin blue.

The nervous system was studied following fixation and staining by the cytological methods mentioned above. Methylene blue was used for tracing the larger nerves. Ranson's pyridine silver, Golgi's mixed method, and Cuajunco's modification of Bielschowsky's technique were the silver methods employed. The following gold-chloride methods were tried:

Löwits, Carter's ('28) modification of Löwits, Ranvier's formic-acid method on fresh material, and, after formol fixation, Ranvier's lemon-juice method, and Viallane's osmic-acid method.

OBSERVATIONS

General organization and behavior of the cilia of the gills

In order to understand the ciliary activity of the gills of prosobranch lamellibranch molluscs, it is necessary to review, briefly, the structure of the gills of this group. Detailed descriptions may be found in the works of Peck ('77), Ridewood ('03), Rice ('06), Field ('22), and others.

Two gills are present on each side of the body, each being composed of an ascending and a descending lamella. The dorsal margins of the two descending lamellae on each side are joined together and are attached also to the body along this line, except in the posterior third; the dorsal margins of the two ascending lamellae lie free in the mantle cavity, except at their anterior and posterior ends. Bands of tissue, which contain blood vessels and connective tissue, are to be found along the free and attached dorsal edges of the lamellae. Each lamella is composed of filaments which extend from this band of tissue to the free ventral edge. The filaments of the inner and outer lamellae are joined by interlamellar connections. The filaments of one lamella are held in normal interrelationship by the action of cilia of the interfilamentous junctions, which are disc-shaped groups of ciliated cells on the sides of the filaments (figs. 1 and 6, *ifj*). A transverse section of a single filament, as seen in figure A, may be described as follows: it has the shape of an oval of which one end is compressed and elongated. The frontal edge bears long cilia, arranged in definite rows, while the abfrontal edge has shorter cilia sparsely distributed. On this edge there are occasionally present long robust cilia (fig. 6, *ac*, and fig. C, *ac*). None are represented in figure A. The cilia of the frontal edge are arranged in three groups: the frontals, the laterofrontals, and the laterals. One or more rows of

glandular cells occur between the laterofrontals and the laterals.

The frontals form a band of epithelium which is four or five cells in width. The active beat of the cilia is toward the ventral edge of the filament. Due to their shortness,

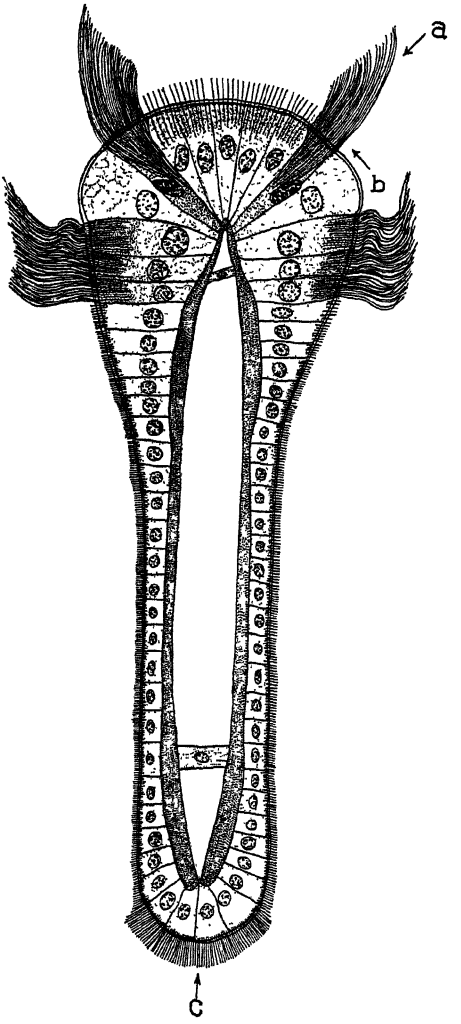

Fig. A Diagram of a cross-section of a gill filament of Mytilus edulis.

these cilia are difficult to study in the living condition and have received little attention in this work. Their function is to move mucus and small particles to the terminal groove on the ventral edge of the filament.

A row of laterofrontal cilia is present on the side of the gill filament (fig. A; figs. 1 and 6, *lfc*). The long axes of the laterofrontal cells are directed at an angle of about 45° with the vertical axis of the filament (Grave and Schmitt, '25). Each cell possesses two rows of cilia. The active phase of the beat is toward the frontal surface, and at the end of the recovery phase the cilium is straight (Gray, '28). The function of the laterofrontal cilia is thought to be that of maintaining the separation of the filaments.

Lateral ciliated cells are found along each side of the filament near the frontal face (fig. A; fig. 6, *l*). These cuboidal cells form an epithelium three or four cells in thickness. The shape of the cells in surface view and the arrangement of the cilia upon them have been described by Engelmann ('80) and by Saguchi ('17). The active beat on the two sides of the filament is in opposite directions, that on the right side is toward the body and on the left side, away from the body.

Below the epithelial covering of the filament, a plate-like layer of chitin occurs which, under low power of the microscope, appears to be homogeneous in structure. It is designated by Ridewood ('03) as a chitinous supporting structure of the filament. An endothelial layer, not easily discernible, rests upon the chitinous structure and bounds the blood space. Cells of various sizes, some of which are known to be blood cells, are found within this blood space. During the course of this investigation, a new type of cell was observed which extends across the blood space at each end, connecting the chitinous structures. It proved to be contractile. In Mytilus edulis contraction of these cells has not been observed during this investigation, but in Modiolus modiolus those on the abfrontal edge were observed to contract and relax.

Normal variations in ciliary activity

Observations and experiments on living material were made in an attempt to determine by the behavior of the living ciliated epithelium whether the influence of a regulatory agent might be evident. For this reason, the normal reaction of the cilia in their natural environment was studied in particular. Hitherto much of the physiological work has been done by varying the external medium; but in this present work it was attempted to keep the external medium constant and to observe the changes in activity arising within the gill tissue itself.

Upon first removal of the gill from the mollusc, there usually followed a stoppage of activity of the lateral ciliated cells, while the laterofrontals continued to beat. These conditions were sometimes reversed, and in some instances, complete stoppage occurred in both. It was seldom that all the laterofrontals of a filament stopped completely; rather, the rate of some decreased, while others became rigid and some showed a flickering at the tips. The laterals were more specific in their behavior and acted more as a unit. A decrease of activity of the laterals was concomitant with a decrease of the velocity of wave propagation and with a depression of frequency, but the wave length remained more nearly constant.

The answer to the question whether these observed changes in activity were due to a change in the surrounding water or to an influence arising within the tissue is important. To determine this point, Modiolus modiolus was chosen for the greater part of the work, because of its thinner mantle and greater resistance to tearing. The mollusc was placed in the observation dish, previously described, and the activity of the cilia was studied with a 4-mm. objective.

Six series of observations were made, each lasting several hours. In the first two a cover-slip was placed over the portion of gill under the microscope and in the subsequent four it was omitted. Even though sea-water flowed over the preparations at the rate of about 6 cc. per minute, it did

not prevent a gradual slowing of ciliary activity, which was undoubtedly due to accumulated waste products. There was gradual decrease in rate without marked decrease in amplitude, which, according to Gray ('22), is the result of increased acidity in the surrounding medium.

Apart from effects due to changes in the external environment, there occurred certain variations in activity which seem to require some other explanation. During one series of observations it was noted that the cilia on all filaments except one within the field of the microscope were beating at their normal rate. All of the lateral and laterofrontal cilia on this filament were completely inactive, and remained so for about a minute. Then a wave of the right lateral cilia passed up the filament. The rate of propagation as judged by eye was somewhat less than that upon the surrounding filaments. The cilia became quiet again for several seconds, and this was followed by sudden activity of all the cilia upon the filament. The change of state drew the filament out of the field of focus, so that the order of activity was not followed, but when next observed, all cilia were beating at the same rate as the others within the field. The medium surrounding all the filaments must have been approximately the same, since surrounding cilia maintained a circulation of sea-water.

A second type of change of activity was noted which also cannot be explained upon the basis of changes in the medium. The laterals, while beating coordinately, show sudden short inhibition periods in which the cilia of one row stop, remain quiet for a second, after which they begin activity again. The stoppage of all the cilia along one row occurs simultaneously, and they all stop in the same phase of contraction. When activity is begun, they may all start suddenly in unison or they may start in small groups. The latter is more often the case, and when this occurs, activity precedes coordination of the movement. When the return to activity is accompanied by uniform movement and coordination, all of the cilia begin in their proper phase relationship and a wave passes down the lateral epithelium. Such sudden short inhibitions may

occur several times a minute. They are very definite and the rate of movement following the reestablishment of co-ordination is the same as before stoppage. It is important to note that inhibitions occur simultaneously at all points on the epithelium within the field of the microscope, indicating that the inhibition impulse progresses down the epithelium at a rate faster than the propagation wave.

A comparison was made of the activities of the cilia when the filament was attached to the mollusc and when it was detached, hoping by this method to make evident any control which the animal might exercise over the movements of the cilia.

Two series of observations were made upon detached filaments of Modiolus modiolus. A description of one series will serve as an example. Several filaments, with the muscle band attached, were placed in a hanging drop. The filaments were in good condition, having few breaks in the epithelium. The laterals had ceased to beat on the greater number of filaments, but on one filament they continued to beat for several hours. During this time groups of laterals on this filament were observed to stop suddenly and to begin beating after a second or two. At the end of one and three-quarter hours, the laterals were all beating, but the wave length was irregular. The blood cells within the blood space showed an oscillating movement in the filament. At the end of two and three-quarter hours, the laterals were more active than before and some were beating which previously had stopped. At the end of five and three-quarter hours, the laterals were beating metachronously and actively, with few sudden stoppages. After six and one-quarter hours, most of the laterals had stopped and disintegration was beginning. The laterofrontals were active throughout the series of observations until the tissue began to disintegrate.

It was soon evident that the cilia showed the same variations in their activity when detached from the animal as when attached. The impulse which produces sudden inhibitions apparently is an intrinsic part of gill tissue.

Microdissection experiments were made in an attempt to determine the nature of the coordination impulse. Filaments of Modiolus modiolus gill were placed in a moist chamber and the microdissection needle was brought up from beneath. A filament in which the laterofrontals had ceased movement, except for a few groups of cilia and for flickerings at the ends of the cilia, was chosen for the experiment. A cut was made through the cuticle between two cilia, as indicated in figure B, and the instant the cut was made the cilia on each side were drawn apart. At the same time a wave passed in the distal direction down the laterofrontal epithelium which

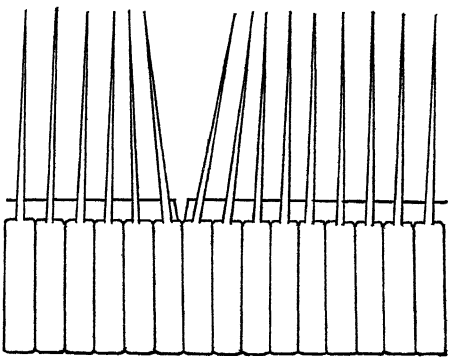

Fig. B Diagram illustrating the cut by a microdissection needle made in the cuticle of the laterofrontal epithelium.

seemed to the eye to travel at the usual rate. Following the initial impulse, the cilia continued to beat actively and at the normal rate. On the proximal side of the cut the cilia began beating, but not as actively or as regularly as is normal, while a little farther along the filament the cilia were motionless. A cut was made through the epithelium between two actively beating cilia, but no effect was observable. The cilia on both sides continued to beat as rapidly as before.

The coordinating impulse of ciliary movement, as discussed later in this investigation, may be more readily understood in the light of tabulations given below. Each of the three variables involved in wave motion, namely, velocity, fre-

quency, and wave length, is listed. The velocity of wave propagation in the laterofrontals and in the laterals was determined by timing with a stop-watch the movement of a wave between two points on an ocular micrometer.

Others methods have been described in the literature (Engelmann, '77; Dixon and Inchley, '05; Inchley, '21; Gray, '24; Umeda, '26) for recording ciliary activity, but the one mentioned above best suited the present purpose.

The observed frequency of vibration of cilia per second was recorded by direct observation. The wave length was derived from the formula $f \times l = v$; in which $f =$ frequency, $l =$ wave length, and $v =$ velocity. Records of laterals and laterofrontals were not taken simultaneously; therefore only the averages are significant.

Table of velocity of wave propagation

Laterofrontals Micra per second	*Laterals* Micra per second	*Laterofrontals* Micra per second	*Laterals* Micra per second
64.3	301.5	72.4	184.9
76.4	201.0	44.2	124.6
64.3	201.0	48.2	116.6
108.5	201.0	60.3	116.6
60.3	201.0	56.3	124.6
40.2	265.3	56.3	144.7
40.2	148.7	56.3	160.8
64.3	132.7	54.3	148.3
72.4	160.8	56.3	168.8
48.2	144.7		
64.3	160.8	Average, 60.3	168.8

Table of observed frequency of vibrations of cilia

Laterofrontals Vibrations per second	*Laterals* Vibrations per second
3.7	7.5
2.9	8.0
3.4	7.5
3.4	9.1
3.9	9.5
Average, 3.6	8.3

Table of calculated wave lengths

Laterofrontals	*Laterals*
15 μ	23.4 μ

From these averages the ratio of each variable of laterofrontal cilia to that of lateral cilia has been calculated, and is presented as follows:

	Laterofrontal		*Lateral*
Wave length,	1	to	1.5
Frequency,	1	to	2.2
Velocity,	1	to	2.8

It is thus apparent that the greatest difference between the two occurs in the velocity and the least in the wave length. In this connection certain measurements upon figures 2 and 3 are significant. The magnification of the two is the same and the ratio of the proportionate number of waves between the lines indicated on the photographs of laterofrontal cilia to lateral cilia is 1 to 1.08.

Cytology of ciliated epithelium

The three views of the laterofrontal cells which have been studied are indicated in figure A. Each view has been given a number for sake of convenience. First, that shown by a cross-section of the filament is termed the side view; secondly, that in the axis of arrow *a*, the frontal view; and, thirdly, that in the axis of arrow *b*, the longitudinal view.

Shape. The laterofrontal cells are tall, columnar, ciliated cells which rest upon the chitinous supporting structure of the filament as seen in figure A. In longitudinal view living cells were found to be of regular rectangular shape, the average width of the cells in Mytilus being 2.6 μ. In fixed material, however, they were found to vary from a rectangular to a triangular shape. When triangular, the apex of the triangle rests upon the chitinous supporting structure. This difference in form of the living and fixed laterofrontal cells is probably due to shrinkage, as is discussed later.

The laterofrontal cell is shown in side view in figure A and in figures 12 and 13.

Teased preparations of the living tissue show the cells to be roughly rectangular, the distal end of the cell being slightly wider than the proximal. In fixed preparations (figs. 12 and

13) the shape is approximately the same. Just below the surface the cell is rather sharply constricted and from that point decreases in width toward the basal region. Due to the large size of the nucleus, the cell is often protruded on one side, as seen in this view. The tapering base of the cell rests obliquely upon the chitinous supporting structure.

Frontal views of the laterofrontal cells were studied at different levels after treatment with the sodium-borate macerating solution (fig. 7a, b, c). In figure 7a, which is a surface view, the cells are rectangular in shape, with the narrow diameter directed in the plane of the epithelium. The ratio of the two diameters of the cell at this level is approximately 1 to 3. Two refractile ridges indicate the location of the basal bodies. In 7b, which is taken at the level just above the nuclei, the cells are irregularly rectangular in shape. In 7c, which is taken through the nuclei, the cells have assumed a definitely triangular shape, which leads to an alternate arrangement of the cells.

Nucleus. The nucleus of the laterofrontal cell has the shape of a compressed oval which lies with its long axis parallel with the long axis of the cell. It averages 8.6 μ in length in Mytilus edulis, and lies within the lower one-half to two-thirds of the cell. The nucleus of the laterofrontal cells of Amblema costata, which were treated with the borax macerating solution, is filled with closely packed but distinct spherical granules, which are refractile in nature. No brownian movement was observed among them. In fixed and stained preparations of both forms the nucleus has a stronger affinity for basic dyes than do the nuclei of other cells of the gill. The basophilic substance of the nucleus is in the form of granules which are irregularly distributed. The nuclear wall is usually distinct, due perhaps to the fact that a small amount of the basophilic substance adheres to it.

Cytoplasm. A clear transparent zone present in many ciliated cells (Saguchi, '17; Merton, '23; Watanabe, '24; de Rényi, '26; Kindred, '27, and others) as a well-defined lightly staining or otherwise differentiated area immediately

subjacent to the row of basal bodies is absent in the laterofrontal cells. The finely granular cytoplasm which is found in fixed material between the ciliary rootlets stains intensely with acid dyes. The remainder of the cytoplasm may be alveolar or reticular in appearance and stains lightly. The cytoplasm of that part of the cell which has not swelled under the influence of the macerating solution also has a granular appearance (figs. 7b, 8, 9, and 15).

In nearly all macerated laterofrontal cells small irregular clumps of refractile spheres are found, which for the present are designated as 'refractile bodies.' They usually lie at the distal end of the nucleus (figs. 8, 9, and 15). These 'refractile bodies' are also seen in the living cells, but as maceration proceeds their number increases (compare figs. 9 and 15). Knowledge of the chemical nature of these bodies is limited to the fact that they are not osmiophilic.

The ciliary rootlets are the most conspicuous structure in the cytoplasm of the fixed and stained laterofrontal cells. The present account differs in some respects from that given by Prenant ('14), Grave and Schmitt ('25), and Bhatia ('26). In longitudinal view (figs. 11 and 14) two well-defined diverging rootlets arise from each of the basal bodies and pass to opposite sides of the nucleus. Though their terminations are indefinite, they disappear in the cytoplasm of the nuclear zone. This observation is strengthened by the appearance of the rootlets in macerated material as seen in figure 15. Bhatia ('26) traced the rootlets to the base of the cell, but his observation is not confirmed by this work. In side view (figs. 12 and 13) the rootlets are very indistinct and indefinite. They lend a faintly fibrillar appearance to the distal cytoplasm. In frontal view the rootlets are seen in cross-section and, though indefinite and frequently appearing to merge, occasionally one is sufficiently distinct to show that each ciliary rootlet is round, and it is therefore apparent that the clear, well-defined appearance of the rootlets in longitudinal view is not due to a greater thickness of the fiber in this direction.

Cell wall. The existence of certain portions of the laterofrontal cell walls has been questioned by Grave and Schmitt ('25), who came to the conclusion that the laterofrontal epithelium is a syncytium. Bhatia ('26) has presented evidence to the contrary, and the present study of macerated and fixed material confirms his conclusion. The macerating fluid at first produces a slight swelling at the basal end of the cell which increases and finally extends through that region of the cell occupied by the nucleus. Such enlargement causes a separation of the cells at the basal portions and an alternate arrangement, as shown in figures 8 and 9 and especially well in figure 7c. The swelling shows clearly that the wall at the base of the cell is continuous with lateral cell walls. After the maceration has proceeded for from two to three hours, the swelling extends to the distal region of the cell, thus making it possible to follow the lateral walls to the cuticle. Moreover, the swelling produced by the macerating fluid occurs when the entire cell wall remains intact. When the cell wall is broken, no new cell membrane forms and the clear fluid which is present within the intact cell wall is absent.

In longitudinal view the lateral wall of the cell may be easily seen in fixed material (figs. 10 and 11) as a well-defined line extending from the surface of the cell to the nuclear zone, where it becomes indistinguishable from adjacent fibers; below the nucleus it is an irregular, poorly defined line, which follows the border of the cytoplasm to the base of the cell. The fixative may cause shrinkage of the cell to the extent that the walls of adjacent cells are separated from each other, producing an intercellular space, as is shown in figure 11. The shrinkage may be so great that the two lateral walls of a cell converge to form a point on the chitinous supporting structure. The coagulated intercellular fluid appears as a faint network of irregularly arranged lines crossing the space in all directions.

Cuticle. Grave and Schmitt ('25) have given a full description of the cuticle. The results of the present investigation differ only in one point. The cuticle is a structure 1.2 μ

in thickness, homogeneous in nature, which covers the end of the ciliated cell with the exception of the small space between the two rows of cilia (figs. 11 and 14). Grave and Schmitt describe an additional break in the cuticle located above the termination of the lateral cell wall. The observations of the present investigation have failed to confirm the existence of such a break; there are areas, however, in these regions which stain more faintly than the surrounding cuticle, but by methods used they are not bounded by definite lines.

Cilia. Carter ('24) and Grave and Schmitt ('25) have described the cilia of the laterofrontal cells. Judging by descriptions of the individual cilium of the laterofrontal cells frequently given in the literature, the cilium seems to assume more clearly defined character when observed in longitudinal view than it does when seen in side view. In longitudinal view (fig. B and figs. 1, 6, 8, 9, 11, and 14) two cilia are seen to arise from each cell. The two converge above the cell surface to form an inverted 'V.' Each cilium appears as a robust fiber. In side view the individual cilium is distinguished only with difficulty and gives the impression of being more slender, as compared with the previous view. In frontal view, which gives a cross-section of the cilia, each cilium is found to be round.

On the basis of the observed arrangement of the cilia as seen in the frontal view of the cell, an explanation is offered in the discussion for the fact that the cilia lend themselves to more satisfactory observation in lateral view.

Since there is a marked difference in the activity of the cilia of laterofrontal and lateral cells, a few general observations upon the cytology of the latter are presented. The lateral ciliated epithelium, which is three or four cells in width, extends on each side along the length of the filament (fig. 6, *l*).

Shape. In frontal view the cells are rectangular with the longer dimension of the cell directed in the plane of the epithelial row. This dimension is approximately the same for most of the cells, and averages 13.2 μ in Mytilus, while the

transverse or shorter dimension is quite variable. In longitudinal view the cells are high cuboidal in shape. In side view (fig. A) they are columnar.

Nucleus. The nucleus is ovate in shape and is located in the lower two-thirds of the cell.

Cytoplasm. The cytoplasm of these cells shows much less marked differentiation than that of the laterofrontal cells. It stains deeply with acid dyes in the supranuclear zone, while in the infranuclear zone it has a fibrous appearance and stains lightly. Under the influence of macerating solutions the cytoplasm resembles that of the laterofrontal cells, but the lateral cells react differently to the solution and do not swell or accumulate a clear fluid in the basal portion of the cell.

The ciliary rootlets are fine, faintly staining fibers which are limited entirely to the supranuclear cytoplasm. They are arranged in perpendicular relationship with the ciliated surface of the cell.

The frontal cells have received very little attention in this work. It may be noted, however, that the cilia which are few in number can be traced through the cuticle and each one is seen to unite to a basal body. From each basal body a single ciliary rootlet passes inwardly perpendicular to the surface of the cell. Along this fiber several nodular thickenings occur. The ciliary rootlets disappear in the nuclear zone. No indication is found of their continuance in the stringy, vacuolar cytoplasm of the basal portion of the cell.

Occurrence and movement of gill muscles

As the investigation of the mechanism of ciliary movement proceeded it became evident that it would have to be extended to include the problem of the entire histological structure of the gill; whether nerves can be demonstrated in the gill and the gill filament; whether the gill structure includes muscle cells, gland cells, etc., that are known usually to require connection with the nervous system for their proper function, and if such tissues are present, whether they or the ciliated epithelium are innervated.

Movement of the entire gill. The shell of a Modiolus was removed, the mantle laid back, and the animal placed upon its side. When forceps were inserted under the edge of the outer gill, partially separating it from the inner gill, the gills themselves extended the separation in both directions. The outer gill so elevated itself that it stood at an angle of about 60° to the inner gill. After rising to this extent, it descended and came to rest on the inner gill. The two gills were again separated with the ends of the forceps and the outer gill elevated itself in the same way, and now continued to rise and fall rhythmically without further stimulation. During these rhythmic movements the elevations usually began at the posterior end of the gill.

Muscle tissue is therefore present, and certain groups of cells located at the dorsal junction of the two descending lamellae were identified as the contractile element involved in the movement of the gills. These muscles are attached to the chitinous supporting structure of each filament.

Muscle cells of the filaments. In Mytilus edulis and in Modiolus modiolus muscles are located in the filaments, and they are arranged in two rows, one near the frontal and the other near the abfrontal edge. Each muscle crosses the blood space and is attached to the chitinous supporting structures, (figs. A and C and fig. 5, *mc*).

These muscles were observed to contract and draw the two sides of the filament together in Modiolus. The arrangement of the muscle cells is shown in figure 6, *mc*. Figure D is an end view of the muscles under high magnification. The figure on the right indicates their appearance in the living unstained tissue.

The muscle cells in figure 5 are approximately square, but often they are slender. Each cell possesses a nucleus. The cytoplasm stains in varying degrees of intensity with acid dyes. In the cytoplasm of fixed material fine fiber-like lines occur which vary in size and number and are distributed in all directions within the cell, but their principal orientation is lengthwise, extending from one chitinous supporting struc-

ture to the other. They seem to embed themselves within this chitin, but this is difficult to determine, due to the fact that the staining reaction of the fiber which is within the chitin is identical with that of the chitin itself. Living filaments of the gill were immersed in a solution of methylene blue, with the result that small blue granules appeared in the muscle cells, arranged in rows which extended lengthwise in

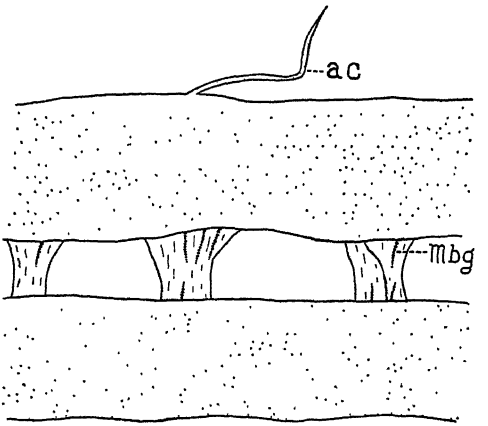

Fig. C Camera-lucida drawing of a gill filament as viewed along arrow *c* of figure A. (Methylene-blue preparation.) *ac*, abfrontal cilium; *mbg*, granules staining with methylene blue.

Fig. D Camera-lucida drawing of the ends of muscle cells.

the cell, usually resting upon one of the chitinous supporting structures (fig. C, *mbg*). No lines of a fiber-like nature were brought out by this method.

Chitinous supporting structure. A layer of chitin which supports the epithelium and a few endothelial cells lines the water space of the gill filaments. In Mytilus edulis it is thickest just below the cilia of the interfilamentar junction and it is also quite thick in the frontal region. The chitinous supporting structure stains blue with Mallory's stain, has

an affinity for toluidin blue, and is light brown when stained with iron haematoxylin. This chitinous structure has been studied carefully to determine whether it might contain nerve fibers or other fibrous elements. It is faintly fibrous and the fibers which are present form a network which in general follows the plane of the filament, giving the filament a laminated appearance (figs. 4, 5, 11, *css*).

The study of the muscles and the chitinous supporting structure has failed to reveal the presence of fibers or other structures which could be definitely identified as nerves.

Gross morphology of the nervous system

If the ciliated epithelium and the muscles of the gill filaments are under nervous control, then it might be expected that a study of the nervous system, and particularly the distribution of the branchial nerve, would reveal the existence and distribution of branches which might enter the gills.

Nerves of the cerebral ganglion. A specimen of Mytilus (shell measurements, 16 × 31 mm.) was fixed in Allen's B_3, embedded in paraffin, and cut into transverse sections 10 μ in thickness. The sections were stained in Mallory's triple connective-tissue stain. The extent of the cerebral ganglion and its nerves in the serial sections was plotted upon graph paper on which each millimeter marking represented 10 μ of the tissue. It was possible in this way to locate and follow their distribution fairly accurately. In Mytilus edulis only three nerves of significant size were found to take origin from each lobe of the cerebral ganglion, namely, the cerebrovisceral connective, the anterior pallial nerve, and a small nerve from the side of the ganglion, corresponding in position to that indicated by Field ('22) as the optic nerve. The first two are large and easily identified, but the small lateral nerve, close to its origin from the ganglion, has a diameter of only 23 μ. The latter nerve takes a course forward, under, and close to the anterior retractor muscle, then turns laterally and lies at the side of the anterior retractor between the muscle and the body wall. Then, after passing forward

through a distance of 0.65 mm., it turns backward and upward and comes to lie close to the body wall. Its first branch is given off after it has passed the anterior end of the gills and is separated from them by a high fold of body epithelium. Each of the branches was followed, one being traced among the liver tubules to the region of a large liver duct, where it was lost among connective tissue and blood cells. The other branch became lost among the liver tubules. Figure 21 is a longitudinal section of this branch of the nerve at the point where it turns upward among the liver tubules. Large spheres colored intensely with the acid fuchsin of the stain are present among the nerve fibers.

It is clear, therefore, than in Mytilus innervation of the gills from the cerebral ganglion, if there be any, must come from the branches of the anterior pallial nerve or the cerebrovisceral connective; therefore the anterior pallial nerve was followed until it entered the mantle. It seemed most unlikely that any branch would turn back through the mantle tissue and enter the gill. Of the eight branches of the anterior pallial nerve that come off between its origin from the cerebral ganglion and its entrance into the mantle, all are small and none are distributed to the gills.

The cerebrovisceral connective was followed through the sections, and it was found that all the branches arising from it are small and that none are distributed to the gills.

Nerves of the visceral ganglion. The visceral ganglion and the nerves arising from it in the fresh-water mussel, Amblema costata, were dissected under the microscope. The animal was immersed in a dilute solution of methylene blue. Though the dye stained only the nerve sheath and not the fibers themselves, it served as a suitable means for tracing all nerves which were large enough to be visible under the dissecting microscope. Figure E is a composite drawing made from

Fig. E A composite drawing representing the nerves which have their origin in the visceral ganglion of Amblema costata. *brn*, branchial nerve; *cs*, crystalline style; *cvc*, cerebrovisceral connective; *g*, gonad; *ibr*, internal branchial ramus; *ilig*, inner lamella of inner gill; *l*, liver; *m*, mantle; *mog*, margin of outer gill; *olig*, outer lamella of inner gill; *os*, opaque substance; *rf*, retractors of foot; *vg*, visceral ganglion; *wp*, water pore.

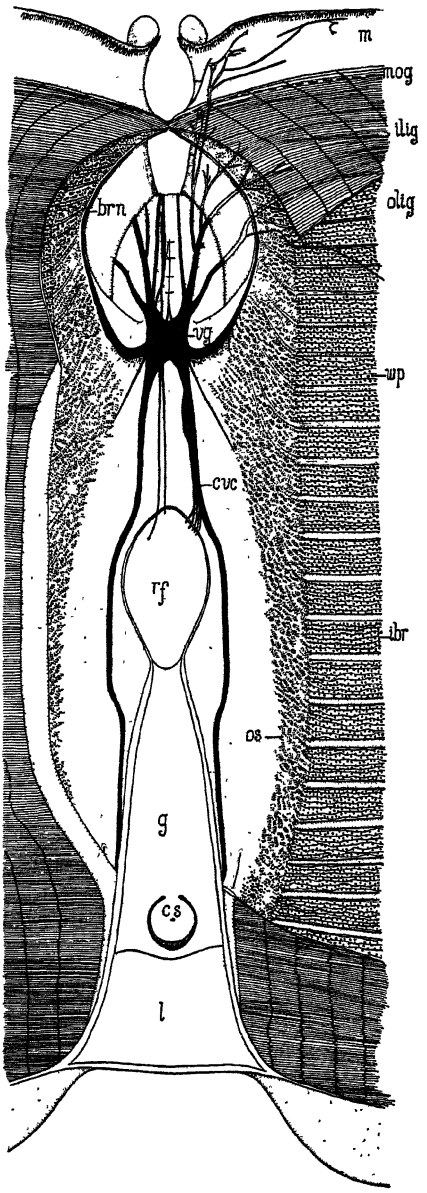

Figure E

the dissection of several animals. The body and foot have been cut away and the gills and mantle spread apart. The right inner lamella of the inner gill has been removed in order to show the inner face of the outer lamella of the inner gill.

The 'H'-shaped visceral ganglion is located on the ventral side of the posterior adductor muscle. The anterior rami are continuous with the cerebrovisceral connectives; the posterior rami give rise to nerves which innervate structures in the posterior region of the animal. The branchial nerve originates from the side of the ganglion.

The nerves which have been traced from the posterior rami are distributed over the ventral surface of the posterior adductor muscle, as shown in figure E. Each nerve and its branches were followed sufficiently far to be certain that no branches were distributed to the gills. The nerves from the anterior rami were followed until the termination of each was evident. None passed to the gill tissue.

The branchial nerve was next dissected. Lucas ('31) has described its distribution and relation to the gills in Mytilus. Splitstösser ('13) has given a clear account of the nerve and its numerous branches in Anodonta.

Effort was made to prepare successful methylene-blue preparations in which it would be possible to follow nerves similar to those described by Splitstösser which enter the gills. Both Amblema costata and Megalonaias gigantea were used, but better results were obtained with the latter. An individual of Megalonaias gigantea (shell size, 13.3 $\times$ 9.3 cm.) was injected through the ventricle of the heart. Some of the fluid passed down the blood vessels which follow the water tubes. The outer lamella of the inner gill was removed at once from the animal and examined. Fibers which had an appearance indicative of nerve structure were found near the inner surface of the gill, lying parallel with the filaments. The filaments are straight, but the fibers follow a sinuous course as they pass around the water pores which open from the ciliated surface of the gill into the water space between the

lamella. The filaments are spaced at intervals averaging 0.089 mm., while the fibers are spaced at intervals averaging 0.124 mm. There are, therefore, 1.4 filaments to one fiber. The diameters of the fibers vary from 0.014 mm. to 0.024 mm., with an average of 0.019 mm. It might be expected that fibers of this size, if they are nerves, could be recognized as such in fixed and stained material, since nerves having a diameter of 8 μ have been recognized and drawn (fig. 21). However, the cross-sections which were examined with especial reference to the identification of these fibers revealed no structures which could be interpreted as nerves. Longitudinal sections revealed no more than did the transverse.

Histology of the nervous system

It was hoped that a more detailed histological study of the lamellibranch nervous system might aid in the location and identification of nerve-fiber terminations. Most of the observations were made upon fixed and stained tissue and upon teased preparations of the living visceral ganglion and cerebrovisceral connective nerve.

Nerve sheath. The ganglia and all the nerves are covered with a sheath which varies in thickness from 1 μ in smaller nerves to 4 μ in ganglia (figs. 16, 20, and 21, *ns*). It stains red with Giemsa's stain and with acid fuchsin and blue with Mallory's triple connective-tissue stain. This sheath is the most resistant element of the nervous system to mechanical pressure.

Nerve fibers. In transverse sections of fixed and stained preparations of nervous tissue, the cut ends of the fibers appear to merge to form a reticulum, and not as independent dots. Likewise, in longitudinal section the individual fibers are indistinct, and irregularly arranged and vacuolar spaces of varying size are scattered among them, which are due to the presence of large homogeneous liquid spheres in the living tissue (fig. 18). In teased preparations the nerve fibers appear as homogeneous refractile lines approximately 0.4 μ in thickness, and there is no indication that they branch or anastomose.

Nerve cells. The nerve cells of the visceral ganglion have been studied in fixed preparations. The layer of ganglion cells lies close beneath the sheath. The largest of the several types of nerve cells present is shown in figures 16, 17, 19, and 20. It is a unipolar cell, approximately oval in shape, and measures about 30 μ in length and from 8 to 13 μ in width. From the centrally directed end of the cell a single nerve fiber arises, while the opposite end of the cell is rounded or pointed and lies adjacent to the nerve sheath.

The contour of the nucleus is not unlike that of the cell itself. The nucleus stains very much in the manner of the cytoplasm, no darkly staining chromatin knots appearing. It contains one large nucleolus, which takes a deep purple color with Giemsa's stain. In some of the nucleoli one or two eccentrically placed areas occur which show a lighter shade of purple (figs. 16, 17, and 20).

The cytoplasm is finely granular and stains somewhat more intensely at the basal end of the cell and in the region at which the secretion spheres are formed (fig. 20). The cytoplasm of the nerve process is a continuation of that within the cell and no indication of any fibrillar structure is found in either (figs. 16 and 19).

The cytoplasm of all nerve cells of this type contains numerous secretion spheres. In the cell represented in figure 17 the central portion of the cytoplasm contains many small, lightly staining spheres. In figure 18 the spheres are larger and fewer in number. In figure 16 a few spheres of large size occur near the margin of the cell and smaller spheres are more centrally placed. Giemsa's stain colors the larger ones a pale greenish yellow and osmic acid blackens them readily. In figure 19 they are concentrated at the base of the fiber against the edge of the cell. Granules of similar form and distribution have been described in nerve cells of other invertebrates by Smallwood and Rogers ('10).

Connective-tissue cells. Connective-tissue cells and fibers are found in the ganglia and larger branches of the nervous system. This cell is oval in shape and is nearly filled by its

nucleus. A fine fiber arises from each end of the cell. The fiber from one end of a connective-tissue cell passes between the nerve cells of the ganglion and becomes embedded in the nerve sheath (figs. 16 and 20, *ctf*), while the fiber from the opposite end passes centrally and becomes lost to view among the nerve fibers.

In teased preparations connective-tissue cells can be identified by the size and shape of the nucleus (fig. 18). Their fibers are the same size as the surrounding fibers and follow the course of the nerve fibers. They are refractile to light. Similar connective-tissue cells and fibers are present among most other tissues of the body and may be present even in the epithelium itself (fig. 4, *ctf*).

Liquid globules. In fixed and stained slides of the nervous system vacuolar spaces occur among the fibers. In the living nerve these spaces are filled with irregularly shaped globules of a clear fluid (fig. 18). If the nerve sheath is broken, these globules flow out into the surrounding medium and there take on a more nearly spherical shape. Alcohol causes a coalescence of the globules, either within the nerve or in the surrounding medium.

Electrical stimulation of the nervous system

Amblema costata is the only mussel used in the series of experiments to determine changes in ciliary activity produced by electrical stimulation of the nervous system. The mollusc was removed from its shell and placed in a dish of tap-water.

Facts concerning the general reactivity of the animal were obtained by stimulating palps, mantle, and foot with a current from the secondary of a Harvard induction coil. The secondary was placed various distances from the primary, which was connected to one dry cell. No muscular contraction in any portion of the animal was produced by stimulation.

On an outer gill which was removed and placed in a flat dish for examination, the lateral cilia were quiescent, but the

laterofrontals and frontals were beating vigorously. The condition of ciliary activity was unchanged by electrical stimulation of the detached gill.

The specimen from which the outer gill mentioned above had been removed was placed ventral side down in the wax-bottom dish. Electrodes were rested on the branchial nerve, and during stimulation the activity of cilia on the gills was observed through the microscope. Before stimulation the lateral cilia were quiescent, the laterofrontal and frontal cilia were active. During and after stimulation, this condition was unchanged; the only effect observed was a slight movement of the whole gill which began from one to three seconds after the beginning of stimulation. Stimulation of the cerebrovisceral connective gave the same results as stimulation of the branchial nerve.

It might be concluded from this work that the nervous system had no influence over ciliary activity, since stimulation of this system produced no change; however, before such a conclusion can be drawn, there must be considered the possibility that removal of the animal from the shell stimulates the nervous system to the extent that further stimulation has no effect.

DISCUSSION

The principal question in this investigation has been whether ciliary activity is controlled by the nervous system. A solution of the problem has been sought through a study of variations in ciliary activity, of the finer structure of the ciliated cells and the tissue around them, and of the morphology of the nervous system.

In each of eight series of observations on the activity of the laterofrontal and lateral epithelia it was found that the cilia slowed in their activity after a variable period of time. It has been suggested above that in some cases this is probably due to accumulation of waste products in and around the tissue. It is known that an increase of acidity decreases the frequency of vibration, but is without effect on the amplitude (Gray, '22 and '28). Also, it has been shown by Hay-

wood ('25) that an accumulation of carbon dioxide without a great increase in acidity will produce the same effect. Behavior of the cilia in some cases, however, cannot be accounted for as due to an accumulation of waste products. In one series of observations one filament possessed quiescent cilia, while those of the surrounding filaments were active. The environment must have been the same for all, hence the cause of the inhibition of activity in this case is thought to have been located within the tissue of the filament. The same reasoning applies to the sudden coordinated inhibitions that occur in the lateral epithelium, to the observed sudden return to activity of the laterofrontal cilia when the cuticle was cut; and to the continued inhibition of the laterals in the fresh-water mussels noted during the electrical-stimulation experiments upon the nervous system.

Elements which might act as conductors for an impulse were first sought in the various component parts of the filaments. The laterofrontal epithelium is not a syncytium, but is composed of cells which are limited on all sides by cell walls, as was demonstrated during their treatment with macerating fluids. The cytoplasm contains two differentiated structures, the basal bodies and the ciliary rootlets. The ciliary rootlets have been demonstrated to extend only as far as the nuclear zone as described by Grave and Schmitt ('25), and not to the base of the cell as described by Bhatia ('26). The lateral ciliated cells possess ciliary rootlets which are perpendicular to the surface and here they also extend only as far as the nucleus. The ciliary rootlets appear clearly defined in longitudinal view of the laterofrontal cells, in comparison to their ill-defined appearance in side view of these cells and in both views of the lateral cells, which is probably due to the fact that the rootlets in the former are arranged in two straight rows and the contrast with the adjacent cytoplasm is greater by observing the edge of a row of rootlets arranged in one plane than is produced by viewing fibrils uniformly distributed through the cytoplasm. The author does not agree with Carter ('28) that a ciliary

rootlet is not a definitely differentiated portion of the cytoplasm. Difficulty in discerning them is not proof of their absence.

Grave and Schmitt suggested that coordination and regulation of ciliary movement take place through the same system of intracellular fibrils. If inhibition of ciliary activity is regarded as an example of regulation, then it hardly seems likely that the impulse for coordination could follow the same pathway that carries the inhibitory impulse, since the coordination impulse in the laterals is found to travel at 168.8 μ per second, while the inhibition impulse travels so rapidly that it seems to stop the cilia in an entire epithelium simultaneously.

A study of the gill filament of the mussel failed to reveal the presence of fiber-like structures which might be considered nerves. The occurrence of muscles in the filaments might indicate the existence of nerves or nerve trunks for their activation, but no connection at the muscles with fibers could be found other than intracellular fibers, and the arrangement and distribution of these are more suggestive of coagulation products than nerves. Thus the examination of the finer structures of the filament by various methods has failed to reveal the existence of nerves.

To ascertain the validity of this negative result, the central nervous system was studied. The conclusion is reached from this portion of the work that the branchial nerve is the only nerve which lies in close proximity to the gills, and it has been shown in Mytilus by Lucas ('31) that the nerve is sensory and that none of the fibers enter the gills. In two freshwater species tissue that stained with methylene blue was found close to the epithelium which lines the water space. These fibers resemble nerves, but studies of fixed and stained preparations of the gill failed to verify this observation. It must be stated, therefore, that thus far no fibers have definitely been traced from the central nervous system to the filaments of the gills.

Following completion of the morphological work, experiments with the living animals were made. The branchial nerve was stimulated with an electrical current, but such stimulation failed to produce any visible effect upon the activity of the cilia.

Evidence as presented in the present investigation points to the conclusion that the impulse regulating ciliary activity of the lamellibranch gill is not dependent upon the nervous system for its transmission.

Therefore coordination and regulation of ciliary activity probably lie within the ciliated epithelium itself, and consideration of certain facts observed concerning the wave movement of cilia may be suggestive on the basis of Parker's theory ('19) of neuroid transmission.

The two rows of laterofrontal and lateral cells lie within the same epithelium, subject to the same general internal and external influences; thus comparison made between the two is valid. The ratio of wave propagation of the cilia on the former to those on the latter is 1 to 2.8, whereas the wave lengths have a ratio of 1 to 1.5 and in the photographs of Amblema gill it closely approaches the ratio of 1 to 1. Thus it appears that between the crests of two succeeding waves a certain distance must exist, whether the rates of propagation and of frequency are high or low. This constancy of wave length is evident also in direct observation of the lateral cilia beating at varying frequencies, resulting from changes in the external medium. Thus the frequency of vibration and the rate of propagation are the variable quantities which tend to be proportional to each other, while the wave length tends to remain constant.

Since the evidence indicates that the cell is conducting a stimulus which passes through its cytoplasm to the adjoining cell in a manner similar to the transmission of an impulse along one nerve process to another, it is reasonable to regard the walls of adjoining cells as synapses in the conduction pathway. It is generally known that transmission across a synapse lowers the conduction time, which may explain why,

in the two rows of ciliated cells under discussion, the rate of propagation is slowest in the epithelium with the greatest number of cell walls per unit length. The ratio of the two propagation rates, 1 to 2.8, is not exactly proportional to the ratio of cell diameters, 1 to 4.9, but, as mentioned above, the same general relationship exists.

In Mytilus the average wave length for the laterofrontal cilia is equal to the average diameter of 5.7 cells and the average wave length for the lateral cilia is equal to the average diameter of 1.7 cells. It is important to note that the wave length is apparently independent of the cell diameter. The cell is not the physiological unit in the production of a ciliary wave, but instead there is involved a certain linear extent of cytoplasm without regard to cell boundaries.

It is evident also that in the lateral cells the impulse is not transmitted to all parts of the cell simultaneously, since all phases of more than half a complete ciliary wave are found in one cell. Since there is progressive activation of cilia within a single cell, it indicates that the impulse enters at the side and passes through the cytoplasm to the opposite cell wall. This being true for the lateral cells, one would expect that the laterofrontal cilia within one cell would also beat at different phase relationships with each other. This, however, does not occur. Instead, all the cilia of one laterofrontal cell beat in unison. Therefore the impulse is probably transmitted simultaneously to all the cilia in one laterofrontal cell in order to produce this result. The explanation upon a physical basis for the progressive activation of the cilia in the lateral cells, in contrast to the simultaneous activation of them in the laterofrontal cells, is suggested by the difference in the arrangement of ciliary rootlets between the two. In the lateral cells ciliary rootlets are perpendicular to the cuticle and parallel with each other, while in the laterofrontal cells two diverging rootlets arise from each basal body and corresponding members from the two bodies converge to a common locus just beneath the cell wall. It is suggested, therefore, that the rootlets on one side of the

nucleus aid in conducting an impulse entering the cell to the basal bodies so that both rows of cilia will be stimulated simultaneously. The rootlets which extend from the basal bodies to the opposite cell wall serve to bring the impulse from each row of basal bodies to the lateral boundary of the cell.

Engelmann ('80) considered it possible that ciliary rootlets correlated the activities of the internal protoplasm and the cilia, but regarded other functions more probable. Grave and Schmitt ('25) also suggested that the ciliary rootlets are involved in the conduction of an impulse. It was their opinion, however, that they served to coordinate the ciliary activity of one cell with the next, but from the present work it is indicated that they are largely concerned with coordination of an impulse within a single cell and have less to do with general transmission of the propagation wave.

Since the coordination impulse seems to pass through the cell cytoplasm, then some other mechanism for conduction of the inhibitory impulse probably exists. This rôle cannot be assigned to nerve fibers, since all work thus far has indicated their absence near the ciliated epithelium. At the present time no other morphological structure can be designated which might be associated with such a function. The inhibition periods do not occur with the regularity found by Carter ('26) in his work upon velar cilia of the nudibranch veliger, so the problem cannot be studied by the methods which he used.

In connection with work on this problem, certain facts have been ascertained concerning the histology of the central nervous system. The large nerve cells of the visceral ganglion elaborate a material in the central region of the cell which appears first as small granules, then it coalesces to form homogeneous spheres of material which have an affinity for osmic acid. This substance may be liberated into the intercellular spaces of the ganglia and nerve, but the substance found in these spaces is, however, less osmiophilic. In living tissue these spaces are filled with globules of clear homogeneous fluid, the surface membrane of which is so affected by

alcohol that they coalesce. It may be that this substance serves the same function as the myelin sheath in the nervous system of vertebrates.

Apparently, the function of the connective-tissue fibers which are found in ganglia is that of support, since one end of one fiber process is attached to the nerve sheath, thus forming a framework for the nerve cells. Usually the nerve cells are separated from each other by connective-tissue fibers, but this is not always the case, many nerve cells having been observed in direct contact (fig. 20).

A detailed study of the nervous system reveals the existence of nerve fibers which in their general structure resemble nerve fibers of vertebrates. Since this is so, it might reasonably be supposed that the termination of these nerves would be fibrous in nature. The presence of such fibers, however, has not been found terminating in or near the ciliated epithelium of the gill.

SUMMARY

1. In the lamellibranch gill variations in ciliary activity occur which are due to other causes than changes in the external medium, and these variations occur as readily in gill filaments detached from an animal as they do in those which are attached.

2. Electrical stimulation of the branchial nerve or the cerebrovisceral connective causes a slight muscular movement of the gills, but has no effect upon the ciliary activity of the frontal, the laterofrontal, or the lateral cilia.

3. From a study of the central nervous system and its branches it seems probable that nerves do not enter the gill filaments, even though in Mytilus and Modiolus contractile cells are present as well as gland cells and ciliated epithelium.

4. The laterofrontal epithelium is not a syncytium, the cells that compose it being bounded on all sides by cell walls.

5. The ciliary rootlets of the laterofrontal and lateral cells terminate in the cytoplasm of the nuclear zone.

6. From a study of wave length, rate of wave propagation, and frequency of vibration of laterofrontal and lateral cilia it is suggested that the coordination impulse passes through the cytoplasm of the cell and that the system of diverging rootlets in the laterofrontal cells serves the purpose of carrying the impulse simultaneously to all cilia within a single cell.

7. The fact that the inhibition impulse travels more rapidly than the coordination impulse indicates that they have different pathways.

8. Several facts concerning the morphology of the lamellibranch nervous system have been added: namely, that the nerve cells of the visceral ganglion form osmiophilic granules, that connective-tissue cells are present in the system, and that the nerve fibers are surrounded by clear liquid spheres. The surface tension of these spheres is lowered by the addition of alcohol.

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EXPLANATION OF PLATES

ABBREVIATIONS

ac, abfrontal cilia
bc, blood cell
css, chitinous supporting structure
ctf, connective-tissue fiber
icf, intracellular fibril
icm, interconnective tissue matrix
ifj, interfilamentar junction
l, lateral cells
lfc, laterofrontal cilia
mc, muscle cell
ns, nerve sheath

PLATE 1

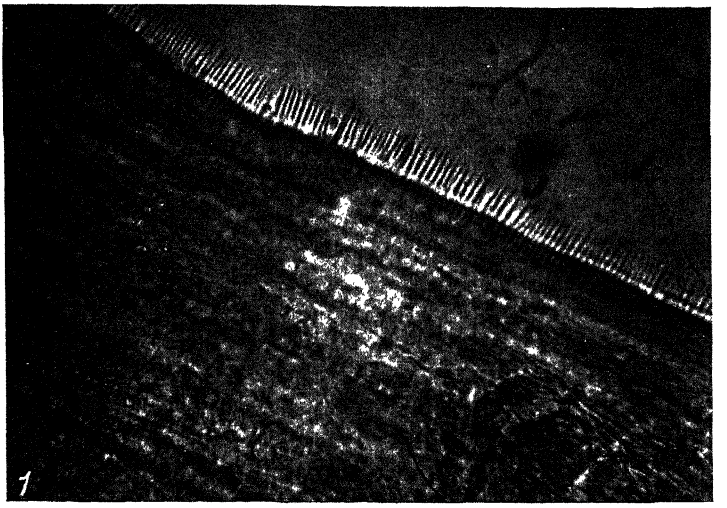
EXPLANATION OF FIGURES

Photomicrographs made with a 4-mm. objective and a 10 × ocular.

1 A portion of a gill filament of Modiolus.

2 A portion of a gill filament of Amblema, to show the wave movement of the lateral cilia. (1/100-second exposure.)

3 A portion of a gill filament of Amblema, to show the wave movement of the laterofrontal cilia. (1/50-second exposure.)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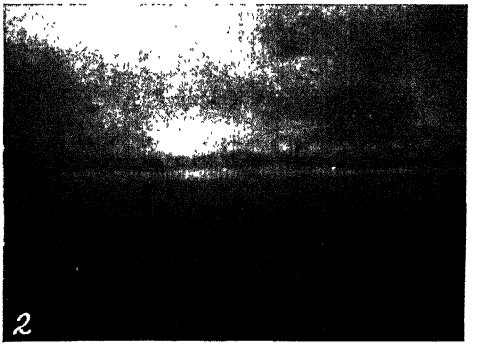
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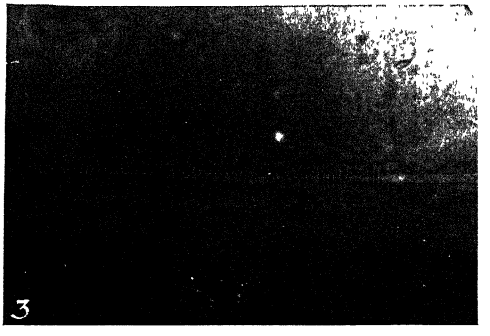
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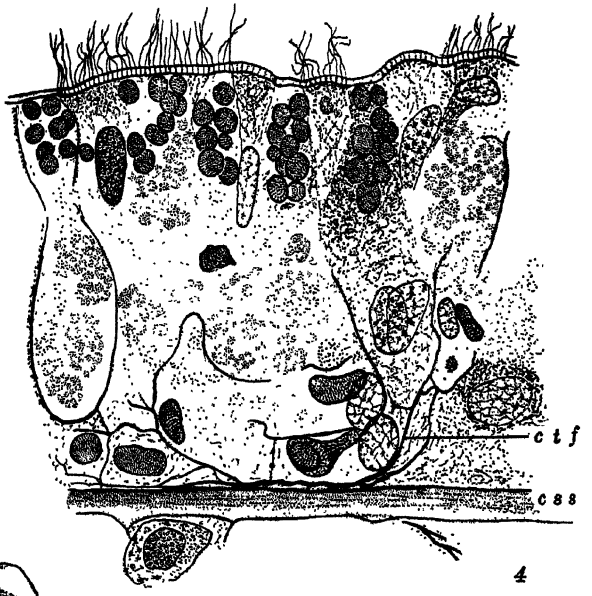
PLATE 2

EXPLANATION OF FIGURES

4 Epithelium near the ventral end of Mytilus gill filament.
5 A section through the abfrontal muscles in a gill filament of Mytilus.
6 Side view of Modiolus gill filament.



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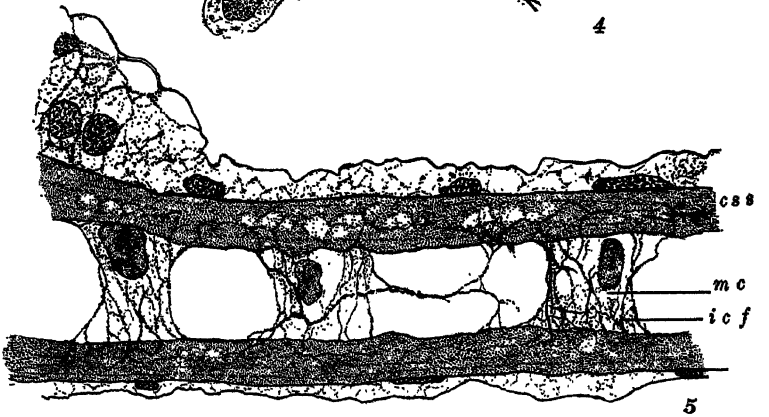

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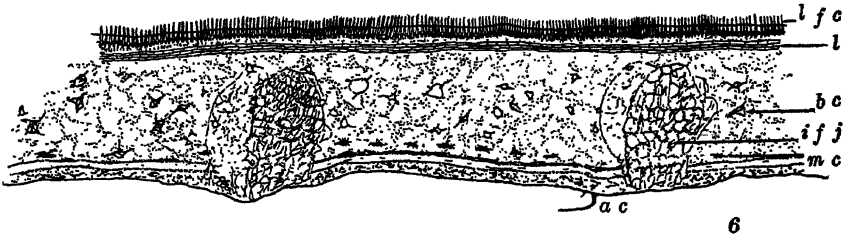

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PLATE 3

EXPLANATION OF FIGURES

7a Surface view of macerated laterofrontal cells from Megalonaias gill.

7b Optical section of the same cells at the level of the refractile bodies.

7c Optical section of the same cells through the nuclei.

8 Side view of macerated laterofrontal cells from Amblema gill.

9 and 15 Side view of macerated laterofrontal cells from Megalonaias gill.

10 Section of laterofrontal epithelium from Amblema gill, so cut that the ciliary rootlets are only partially included.

11 and 14 Section of laterofrontal epithelium from Mytilus gill.

12 and 13 Cross-section of laterofrontal epithelium from Mytilus gill.

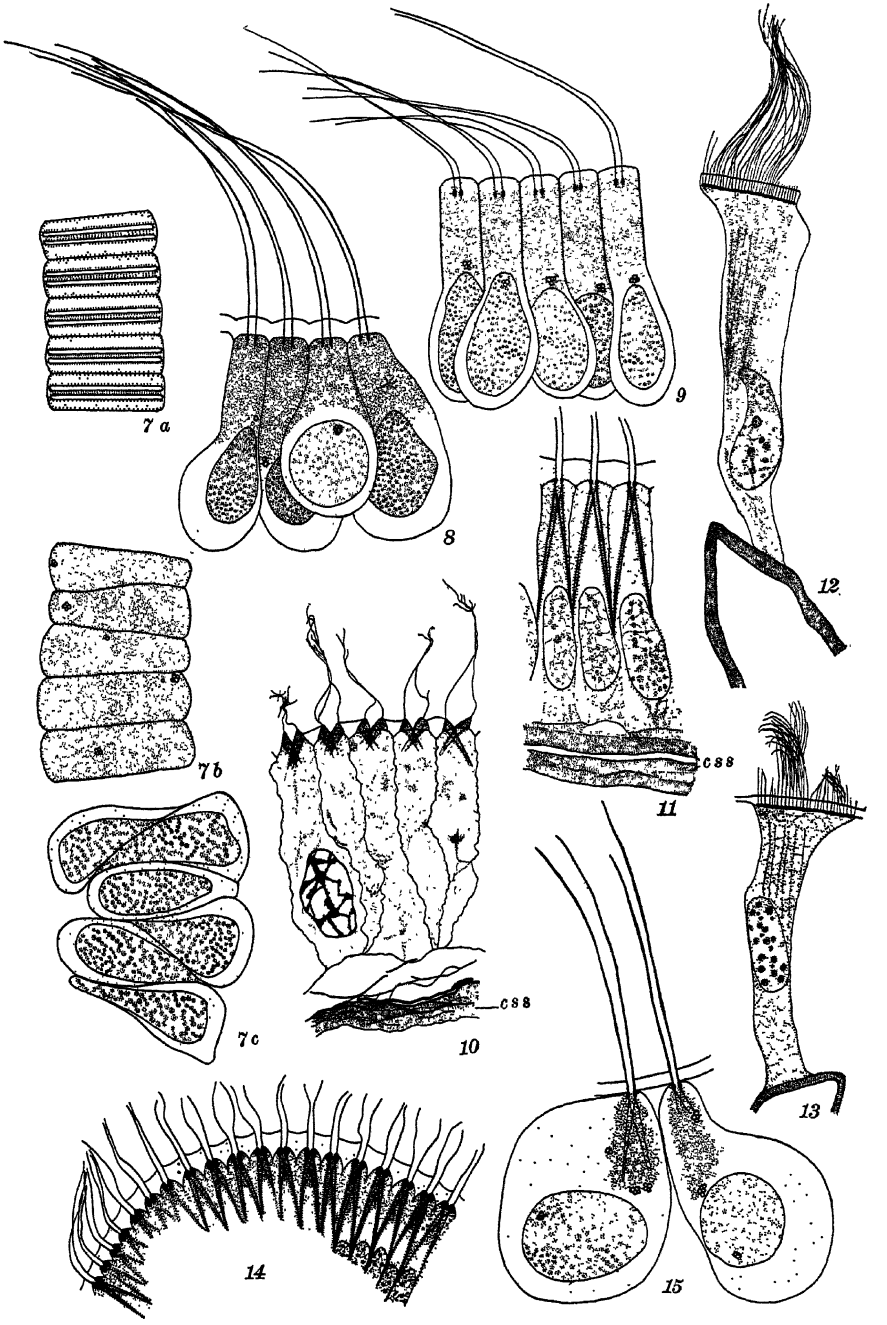
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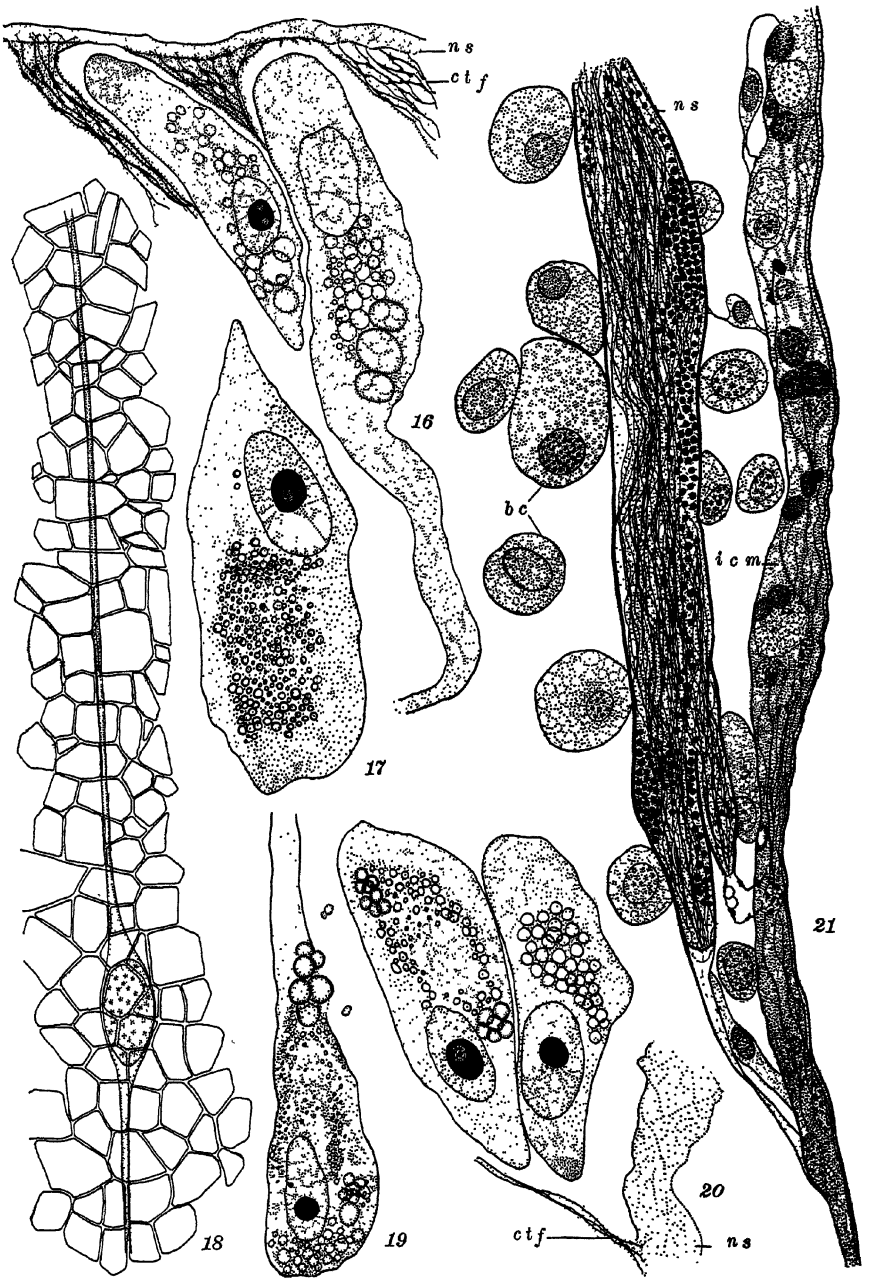
PLATE 4

EXPLANATION OF FIGURES

16, 17, 19, and 20 Nerve cells from visceral ganglion of Amblema costata.

18 Connective-tissue cell surrounded by liquid globules from teased preparation of cerebrovisceral connective of Amblema costata.

21 Mytilus. Longitudinal section near the termination of a nerve which arises laterally from the cerebral ganglion. Interconnective tissue matrix supporting the body-wall epithelium lies to the right. Several types of blood cells are shown.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THE DISTRIBUTION OF THE BRANCHIAL NERVE IN MYTILUS EDULIS AND ITS RELATION TO THE PROBLEM OF NERVOUS CONTROL OF CILIARY ACTIVITY

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ONE PLATE (EIGHT FIGURES)

AUTHOR'S ABSTRACT

The branchial nerve of Mytilus edulis, traced by means of serial sections, has been found to be limited entirely to the epithelial and connective tissues bordering the axis of the gills. Most of the numerous branches which originate from the branchial nerve extend posteriorly and lie close to the interfibrillar matrix of the connective tissue which supports the epithelium of this region. Fibers of these nerves have been traced to this epithelium.

The chitinous supporting structures of the gills lie in close proximity to these nerves, yet neither nerves nor nerve fibers have been observed to penetrate them. Moreover, a careful study of the gill tissues fails to reveal the presence of structures which might be interpreted as nerves or nerve fibers.

Since no innervation of the gills has been demonstrated, it seems probable that the ciliary activity of the gill epithelium is not regulated by means of fibers connected with the central nervous system.

INTRODUCTION

In lamellibranchs ciliated epithelia are present on nearly all exposed surfaces of the body, the lining of the digestive tract, and the ducts of the reproductive organs. These animals are dependent upon the movement of these cilia for food, for conveyance of respiratory gases, for transport of sexual products, and for the cleansing of the body. Some direct regulatory control over motile organs of such importance might be expected, and since the animal possesses a central nervous system, it seems reasonable to search for a morphological relationship between the two.

The experimental work of Copeland ('22) indicates that the nervous system may be involved in regulating ciliary activity on the foot of the snail Polinices. Carter ('26) conducted a series of experiments which demonstrate conclusively that the inhibition of ciliary movement in the velar cilia of

the nudibranch veliger is due to impulses carried by the central nervous system. He described ('28) the termination of these nerves in the cytoplasm of the ciliated cells.

The gill of the salt-water mussel, Mytilus edulis, is particularly favorable material for the investigation of this problem, because it has been used in many physiological studies of ciliary movement. It is relatively simple and unspecialized in structure, according to comparisons made with gills of other animals by Ridewood ('03). The subfilamentar tissue which is characteristic of nearly all the lamellibranchs is absent in Mytilus and other members of this family, and, finally, the gills have relatively few points of attachment to the body.

I wish to thank the Zoölogical Department of the University of Kentucky for the privilege of using the laboratory during the summer of 1929. I am indebted to Prof. Caswell Grave for very helpful criticism of the manuscript.

MATERIAL AND METHODS

A Mytilus, 16 × 31 mm., was sectioned serially and treated with Mallory's triple connective-tissue stain. The nerve cells and fibers and connective-tissue fibers are colored red, while the interconnective tissue matrix which surrounds the nerves and lies subjacent to the epithelium stains blue. By means of this color contrast, the nerves may be followed to structures within which they terminate.

OBSERVATIONS

Three nerves arise from the cerebral ganglion, of which one extends anteriorly, one laterally, and one posteriorly. Each of the three has been traced, and it has been found that no branches enter the gills.

The anterior pallial nerve is distributed mainly to the anterior portion of the mantle, as described by Purdie ('87) and by Field ('22). No recurrent branches were observed to approach the gills.

The lateral branch, which has been described in a previous paper (Lucas, '31), apparently terminates among the tubules of the liver.

The cerebrovisceral connective, springing from the posterior face of the cerebral ganglion, gives rise to numerous small branches, but none are distributed to the gills.

Because the pedal ganglion is relatively far removed from points of attachment between body and gills, its connections have not received such careful study as that given to other parts of the central nervous system.

From the visceral ganglion, lying beneath the posterior adductor muscle, those nerves originate which in general innervate the posterior parts of the body. Only one of these, namely, the branchial nerve, lies in close proximity to the gills. This nerve arises from the ventrolateral border of the ganglion, turns in a posterior direction almost immediately, and follows the dorsal margin of the descending gill lamella. It is described in most accounts as a single nerve which extends to the posterior extremity of the gill. Field ('22) states that it gives rise to numerous fine branches which pass in an anterior direction along the axis of the gill. Their terminations are not discussed. Splitstösser ('13), using Anodonta, has also described branches arising from this nerve and has traced them into the descending lamella of the gills, but in the species with which he worked a well-developed subfilamentar layer is present which is altogether lacking in those forms like Mytilus possessing a simple gill structure, and it is into this subfilamentar tissue that he traced the branches of the branchial nerve. As reported by Lucas ('31), Splitstösser's observations have been confirmed in Megalonaias gigantea, a form similar to Anodonta, but in neither Splitstösser's nor in the author's study has it been possible to trace these branches into that region of the gill which corresponds to the gill of Mytilus edulis or Modiolus modiolus, namely, the region of the filaments which bear the cilia.

Figures 1 to 6, drawn from sections through the dorsal margin of the gill of Mytilus, show the general relationship of organs and structures that lie between the regions of the visceral ganglion and the posterior tip of the gill. Branches arise from the median, ventral, and lateral sides of the branchial nerve (figs. 1 to 4, *BR.N.*), which soon turn and extend in a posterior direction. They lie close to the epithelium, separated from it by a layer of interconnective tissue matrix.

The distribution of these nerves is fairly localized, although they are not excluded from any portion of the tissue supporting the gills. Nerves are present on both sides of the gills within the angles which occur at the junctions of the descending lamellae and the tissue supporting them. On the median side (the left side of figs. 1 to 4, the right side of fig. 5) a single nerve is usually found which is well defined and easily distinguished from the adjacent tissues. It is shown in figure 8 under high magnification. On the opposite side, where the angle is very obtuse (figs. 1 and 2), the nerves are composed of numerous branches, but posteriorly (figs. 3 to 5), where the angle is small, a single one is present. A contributing nerve to this group is shown in the central portion of figure 2, and this particular one before it approaches the epithelium divides into several branches. The transverse portions of such nerves have been identified in gross dissections. Just anterior to the point at which the gill separates from its attachment to the body, several large branches, of which one is shown in figure 4, arise from the branchial nerve. They either join the group of nerves previously described or lie below the epithelium in the same horizontal plane as the branchial nerve (fig. 5).

Most of the epithelium covering the region in which these nerves lie is composed of irregular cuboidal cells (fig. 8) whose walls and free borders are difficult to identify. Some cells contain secretory granules. Two portions of the epithelium are differentiated into sense organs, the osphradium on the median side near the margin of the branchial nerve (figs. 1

and 7, *O.*) and the abdominal sense organ on the lateral side (fig. 3, *A.S.O.*). Both are distinguished by columnar cells, whose walls are fairly well defined, because the cytoplasm stains lightly. Field ('22) has reviewed the literature concerning their structure and function. Numerous branches from the visceral ganglion and the branchial nerve extend to the osphradium. The nerve supply to the abdominal sense organ cannot be definitely traced, but in figure 3 a large branch is shown, which as it approaches the lateral epithelium disappears in the interconnective tissue matrix which is abundant in this region. The character of this matrix which extends to the sense organ suggests the presence of nerve fibers, since its finer structure is that of the matrix which lies subjacent to the osphradium and other parts of the epithelium in the region suspending the gills. Because of their small size and great numbers, individual fibers have not in this case been successfully traced to their terminations.

Part of the gill is freely suspended in the mantle cavity (fig. 5), but the posterior tip is attached to the mantle by means of areas of interlocking cilia (fig. 6, *C.J.*). All nerves decrease in diameter toward the end of the gill and disappear before the tip is reached. Moreover, the posterior pallial nerve which lies in the mantle tissue does not give rise to any recurrent branches which might enter the gill through its posterior end.

Although nerves are numerous above and at both sides of the gills, none have been observed to enter the gills. Small branches may even lie upon the chitinous supporting structure near the junction of the two lamellae, yet they have not been found to penetrate it. These observations alone do not preclude the possibility that they enter as separate fibers. A detailed study of the distribution of these nerve fibers was made and a comparison instituted between the interconnective tissue matrix which bears these fibers and the chitinous supporting structure of the gill.

In order to understand more clearly the description of the nerve fibers, it is necessary to give a brief account of the

interconnective tissue matrix in which they are found. The matrix, which is colored blue with Mallory's triple connective-tissue stain, forms the sheath covering the ganglia and the nerves. It also forms the loose supporting substance between organs and structures, and is present as a compact layer of varying thickness beneath the epithelia of the body. It may have well-defined limits or it may merge from a dense layer to an open network. The various states of this tissue are represented in the figures of plate 1, *I.C.M.* Blood cells, connective-tissue cells, and others have been found in the meshes of this network. It is possible that isolated nerve cells are also present, but they cannot be definitely identified. It is generally true that a larger amount of matrix is associated with nerves from which fibers are emerging than from those not so branching. This may be present as a layer of increased thickness between the epithelium and the nerve or as a compact strand lying parallel to the nerve and in close proximity to it. Cross-sections of the latter are represented in the lower part of figure 4.

The nerve-cell bodies, which in general are grouped near the periphery of the ganglion and of the branchial nerve near its origin, are especially numerous on the sides of these structures toward the epithelium, and they occur also within the nerves distributed to the osphradium (figs. 1 and 7, *N.C.*). Other nerves, such as the one shown in the upper part of figure 7, may not be so well supplied with nerve cells, but all contain at least a few, even in parts far removed from the ganglion (fig. 8).

The matrix upon which the epithelium rests is characterized by the presence of many canals and fibers, which are usually arranged parallel to the plane of the epithelium (figs. 7 and 8, *C.* and *C.T.F.*). The canals are not all alike nor are all the fibers of the same sort. The former are spaces in the matrix with diameters of about 0.6 μ, whose walls are not differentiated from the surrounding substance. Because these spaces are relatively long and approximately circular in cross-section, the term 'canals' has been applied. Many

have their origin from the margins of nerves, then turn and run parallel to similar canals, or they may extend directly toward the epithelium. The material within the canal is continuous with the substance of the nerve tissue and is similarly stained, but less intensely. Therefore it seems likely that these structures represent nerve fibers passing through a connective-tissue matrix. Their diameter is so small that not much has been learned concerning their finer structure. Small granules may occasionally be seen, but nothing to indicate the presence of neurofibrils. In these preparations, however, fibrils are absent in all nerves, even at their origin from the cell bodies. There is probably but one fiber in each canal. Their final termination is the epithelium and numerous cases have been seen in which the fiber joins these cells (figs. 7 and 8). The error in the identification of this termination is small, because the fibers, except where they enter the epithelium, are always separated from it by some of the blue-staining matrix, although it may be even thinner than the diameter of the fiber. The nature of the final endings of the fibers among these cells has not yet been determined.

A second type of fiber is found in the matrix which might easily be confused with the one just described. It also stains red, but more brilliantly. It is definite in outline, constant in diameter, homogeneous in structure, and wavy in form (figs. 7 and 8, *C.T.F.*). In general, a fiber of this kind follows a canal of the matrix, but is not definitely limited to it. The amplitude of some waves may exceed the limits of the canal and in some cases a canal may be lacking (fig. 8). Moreover, none have been traced to the epithelium, so by their appearance, staining reaction, and similarity to connective-tissue fibers in other parts of the body, it is probable that they are to be identified with connective tissue.

It has previously been stated that the larger nerves do not penetrate to the gills, and now it must be determined whether the same is true of the fibers. Figure 8 is a highly magnified section of the region similar to that represented

in the lower left-hand part of figure 2. It includes epithelium, matrix, a section of the nerve which lies in the angle between the junction of the epithelium and the gill, and a portion of the chitinous supporting structure. The matrix, which contains a large number of canals carrying nerve fibers, shows numerous examples of continuity between a large nerve and the substance within the canals. The chitinous supporting structure of the gill stains blue, as does the matrix, but structurally it is very different. It is homogeneous, contains no canals and no fibers that can be regarded as nerves. A marked and consistent contrast is found between the matrix which contains nerve fibers and the chitinous supporting structure which does not. Occasionally, a wavy connective-tissue fiber is found within the chitinous supporting structure, but it corresponds to non-nervous fibers previously described and neither its origin nor its distribution suggests a connection with the nervous system.

DISCUSSION

The branchial nerve and its branches, which are limited altogether to the tissue suspending the gills, transmit their fibers through the interconnective tissue matrix to the epithelium. Apparently, most of the epithelium, as well as two sense organs, the osphradium and the abdominal sense organ, are well supplied with nerves. This, coupled with nearly complete absence of muscles, indicates that the function of the branchial nerve is mostly, if not entirely, sensory.

What may be the rôle in the economy of the animal of such an area of sensory epithelium is difficult to determine, but the close connection of the epithelium with the central nervous system by means of such a relatively large nerve supply indicates that the sensations are of more than local importance to the animal and that the impulses are transmitted to more distant parts of the body.

It has been found that the branchial nerve which lies close to the gills does not send branches to that organ and so obviously cannot innervate its ciliated epithelium. The

ciliated epithelium is not the only contractile organ of the gills which must function without central control, for, in a previous paper, Lucas ('31) describes muscles that extend across the blood spaces.

By means of the technical methods used thus far, it is tentatively concluded that the branchial nerve is sensory in function, that the gills are autonomous organs, and that the ciliated epithelium is not regulated by the nervous system.

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PLATE 1

EXPLANATION OF FIGURES

The figures have been outlined under a camera lucida from fixed and stained preparations of Mytilus edulis. Figures 1 to 6 were magnified 129 diameters and figures 7 and 8 were magnified 1200 diameters before reduction in publication, which was one-third. The median line of the animal is toward the left side of the figure, except the fifth, and in this case it is toward the right.

1 Section through the visceral ganglion, *V.GL.*, at the origin of the branchial nerve, *BR.N.*

2 Section 0.87 mm. posterior to figure 1.

3 Section 1.54 mm. posterior to figure 1, through the abdominal sense organ, *A.S.O.*

4 Section 2.12 mm. posterior to figure 1, near the point of separation between the gill and its attachment to the body.

5 Section 3.80 mm. posterior to figure 1, through a dorsal border of the gill which is free in the mantle cavity.

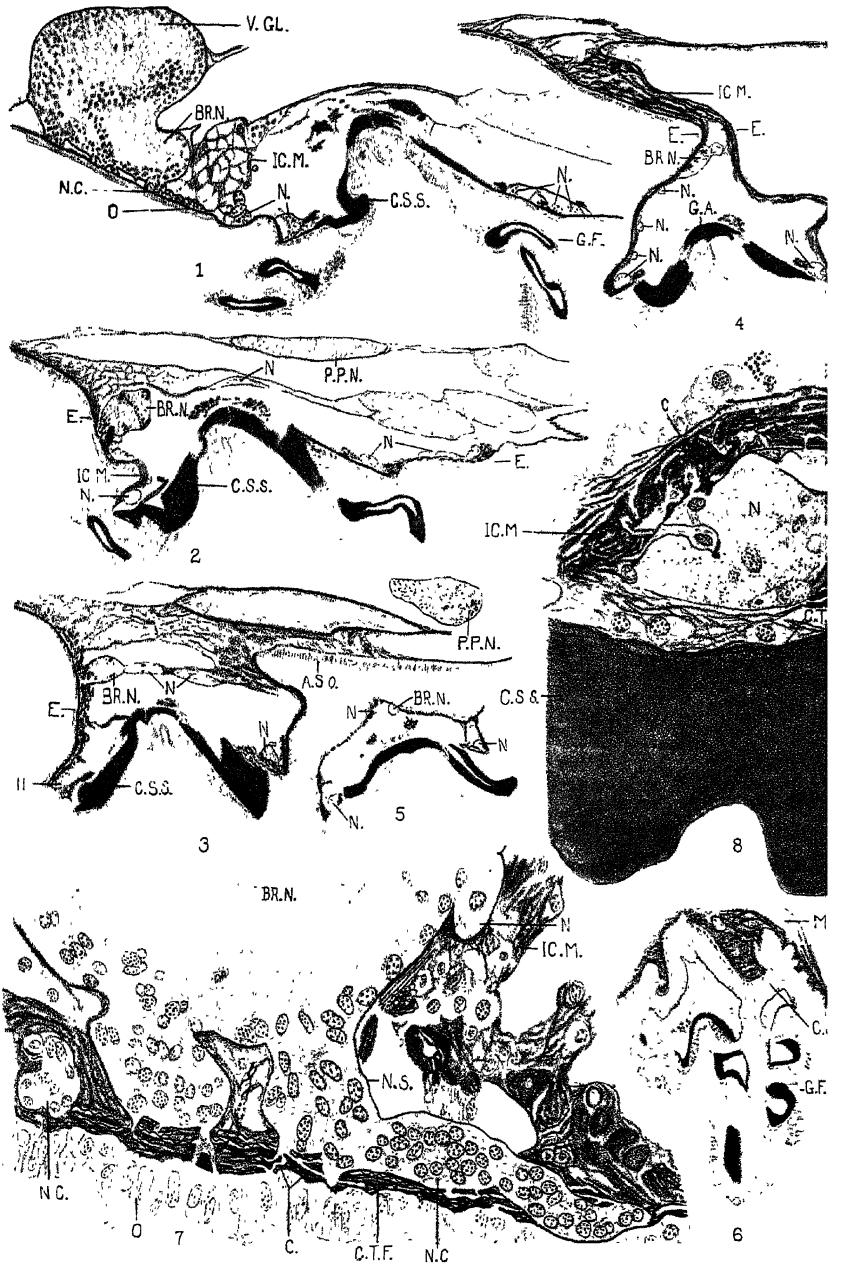
6 Section 4.82 mm. posterior to figure 1. The gill is attached to the mantle by means of interlocking cilia.

7 Section 0.29 mm. posterior to figure 1, through the branchial nerve, *BR.N.*, and osphradium, *O.*

8 Section 1.24 mm. posterior to figure 1, through a nerve, *N.*, between the gill support and inner descending gill lamella.

ABBREVIATIONS

A.S.O., abdominal sense organ
BR.N., branchial nerve
C., canal containing nerve fiber
C.J., ciliary junction
C.S.S., chitinous supporting structure
C.T.F., connective-tissue fiber
E., epithelium
G.A., gill axis
G.F., gill filament
I.C.M., interconnective tissue matrix
M., mantle
N., nerve
N.C., nerve cells
N.S., nerve sheath
O., osphradium
P.P.N., posterior pallial nerve
V.GL., visceral ganglion


V. GL.
BR.N.
IC.M.
N.C.
O
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C.S.S.
G.F.
1
IC M.
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BR N.
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6

THE LABYRINTH OF BUFO VULGARIS JAPONICUS LARVA. ESPECIALLY, UPON THE MORPHOLOGIC STUDY OF THE PERILYMPHATIC SPACE, IN CONNECTION WITH THE MEMBRANOUS LABYRINTH AND AUDITORY CAPSULE

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TEN PLATES (THIRTY-TWO FIGURES)

AUTHOR'S ABSTRACT

A morphologic study of the labyrinth, especially on the perilymphatic space with its physiologic aspect, is presented in this paper. The perilymphatic space starts its development with the chondrification of the auditory capsule, and is completed by the end of the first third of metamorphosis. The author divides the whole spatium into two parts: the ductus perilymphaticus et diverticula and the pars spongiosa spati perilymphatici. The ductus perilymphaticus et diverticula may play an important rôle in carrying out the functions of both equilibrium and audition. The pars spongiosa serves not only to fasten the membranous labyrinth to the capsular wall, but acts as a safeguard for the functions of both the membranous labyrinth and the ductus perilymphaticus et diverticula. The ductus system may have more important physiologic relations than does the membranous labyrinth in connection with the cranial cavity and spinal cord, as to the change of pressure, the transmission of vibrations, the osmosis of fluids, etc. A number of microscopic and schematic figures are shown with reference to the anatomic and morphologic relations of the membranous labyrinth, auditory capsule, and spatium perilymphaticum.

CONTENTS

Introduction and history 207
Material and methods 211
Observations 213
A. Labyrinthus membranaceus 215
B. Capsula auditiva 218
C. Spatium perilymphaticum 221
1. Ductus perilymphaticus et diverticula 221
2. Pars spongiosum spati perilymphatici 224
Summary and conclusions 226
Bibliography 229

INTRODUCTION AND HISTORY

The internal ear, or membranous labyrinth, is known to us as a small, delicate sense organ which is concerned with audition and equilibrium. In the Amphibia it consists of two saccules and three semicircular attachments, and is anchored to

the inner wall of the auditory capsule by a network of fibrous tissues. The space between the labyrinth and the capsule, partly filled with a fibrous tissue, is generally called the perilymphatic space, and is pervaded by a watery fluid called the perilymph, which is like the endolymph which fills the membranous labyrinth. The free space between the labyrinth and the auditory capsule forms a definite, constant, and restricted duct-like cavern which takes a complicated course around the membranous labyrinth, connecting finally with the subarachnoid space in the cranial cavity. This canal system is called the ductus perilymphaticus, of which the anatomic and morphologic structure has been more considered than the other part of the perilymphatic space. Yet the perilymphatic space as a whole has been rather neglected and less investigated than any other part of the labyrinth, as the membranous labyrinth. For this reason, the author has chosen to study the labyrinth of the Bufo vulgaris japonicus larva, especially the morphological relations of the perilymphatic space, with the membranous labyrinth and auditory capsule.

On the perilymphatic system of the Amphibia, especially the Anura, such scholars as Hasse ('73), Kuhn ('80), Retzius ('81), Villy ('90), Harrison ('02), Gaupp ('04), and Hane ('14) have written. They worked mostly on Rana fusca (temporaria), Rana esculenta, and, rarely, Bufo cinereus and vulgaris. Hane studied Bufo vulgaris japonicus.

Hasse ('73) first described how the perilymph communicates with the peripheral lymph vessels which later join the cavum epicerebrale by the two ducts of the cavum perilymphaticum and saccus perilymphaticus.

Kuhn ('80), in his study of the amphibian ear, recognized the existence of the ductus perilymphaticus described by Hasse, and added that the 'liquor perilymphaticus' probably circulated in this short cylindrical duct.

Retzius ('81) studied the perilymphatic space of Proteus anquinus. He differentiated between the ductus and the space filled with the meshwork of fibrous tissue. He described further in detail the ductus of Bufo vulgaris Lauer, as follows:

Dieser Raum ist am weitesten an der lateralen Seite des Sacculus und Lagena; von hier aus steigt er, ebenfalls räumlich, vorn an der lateralen Seite des Utriculus empor, wird dann, an der lateralen Seite des Sinus empor, nach oben und aussen vom hinteren Ende des äusseren Bogengangs, enger, röhrenförmig, cylindrisch, und liegt sich, zum Ductus perilymphaticus werdent, lateralwärts vom hinteren Ende des äusseren Bogengangs, nach hinten und dann nach unten um; dann sendet der Gang, lateralwärts vom Sinus posterior liegend, nach unten-hinten, biegt sich, unter dem letzteren und hinter der Pars neglecta hinziehend, nach innen hin, um, stets eng röhrenförmig, in der dreieckigen Partie zwischen den letztgenannten beiden Bildungen, dem Ramulus ampullaris posterioris und der Pars basilaris an der medialen Seite des Gehörorgans hervorzutreten. Hierauf biegt sich der Gang nach hinten um und erweitert sich ziehmlich stark, endlich biegt er sich wieder, nach Bildung einer kleinen Ausstülpung, einen kleinen Saccus perilymphaticus, nach innen um geht durch die in der medialen Gehörkapselwand belegene Apertura ductus perilymphatici (Aquaeductus cochleae) in die Schädelhöhle hinein, um sich in die innere Hirnhaut fortzusetzen.

Besides, he mentioned for the first time the ductus fenestrae vestibuli, projecting from the saccus sacculare into the fossa fenestrae vestibuli.

Villy ('90), who studied Rana fusca from the descriptions of others, called attention to the independent wall of the vestibulum which is in direct contact with the perilymphatic system. He stated that the ductus perilymphaticus reached the pars basilaris after first entering the saccus perilymphaticus through the cranial cavity, but he mistook the pars basilaris for the lagena.

Harrison ('02) published many conclusions as the result of his thorough investigations on the perilymphatic spaces of the amphibian ear, both of the Anura and Urodela. On the morphological and physiological aspect, he emphasized that the 'tympanal areas,' as he called the independent walls on the vestibulum in contact with the perilymphatic system at the spatium sacculare, recessus partis basilaris, and recessus partis neglectae, were situated in the opposite sides of the maculae acusticae, and thus the vibrations of the perilymph in the ductus would be transmitted to the endolymph, and would naturally stimulate the nerve peripheries of the macu-

lae. (In the pars neglecta Asai ('18) called the macula the crista neglecta, according to his histological study.) The vibrations from the membrana fenestrae vestibuli, as he explained, would be transmitted by the perilymph of the ductus, first to the tympanal area of the sacculus; then, after proceeding along the duct, they would reach the pars neglecta at the recessus partis neglectae, and finally, after passing through the saccus perilymphaticus in the cranial cavity, they would reach the recessus partis basilaris. He found that the recessus partis basilaris was an independent short projection from the saccus perilymphaticus into the auditory capsule through the foramen perilymphaticum inferius, denying Hasse's description of it as a part of the direct course of the ductus into the cranial cavity. According to his study, these relations were quite similar in Rana fusca and Bufo, while the recessus partis basilaris of the former was not communicated with the cranial cavity before it reached the foramen jugulare, and the saccus perilymphaticus seemed more important than that of the latter.

Gaupp ('04), in his great work, "Die Anatomie des Frosches," described the perilymphatic spaces according to his own investigations and the works done by Retzius and Harrison. Gaupp divided the perilymphatic spaces into two, the upper smaller and the lower greater portion. The latter was the large space which occupied the lateral and posterior side of the sacculus, lagena, and pars basilaris as far as the lateral wall of the auditory capsule, although in young larvae it was undeveloped and very small. The upper line reached the dorsal edge of the sacculus, extending from the anterodorsal to the posteroventral side. The upper smaller space was located at the angle of the utriculus and sacculus, forming a wide duct, the pars communicans, which extended upward as far as the recessus utriculi. He agreed with Harrison on the route of the ductus, but described the pars neglecta as projecting into the ductus, and overlooked the existence of the recessus partis neglectae.

Hane ('14) divided the entire ductus system of the Bufo vulgaris japonicus into six parts, viz., the spatium perilymphaticum, spatium lagenae, saccus fenestrae vestibuli, ductus perilymphaticus, saccus perilymphaticus, and spatium meningeale. The spatium perilymphaticum was the largest of all, in close contact with the sacculus; the spatium lagenae, found by himself, at the anteromedial side of the cranial wall of the sacculus, communicated with the spatium perilymphaticum at the ventral edge of the sacculus and lagena; the saccus fenestrae vestibuli (Retzius) was located at the lateral side of the spatium perilymphaticum, with which it communicated through the fenestra vestibuli. On the problem of the recessus partis basilaris, he agreed with the description by Harrison, instead of those of Hasse and Kuhn.

Although our predecessors, as mentioned briefly above, have already more or less studied the perilymphatic spaces of the Amphibia, especially the Anura, these narrow spaces have not yet been thoroughly described. Their descriptions sometimes differ from one another and they set forth erroneous results, on account of the complexity of the anatomic structure and the difficulty of obtaining good serial sections followed by reconstructions. Therefore, the author, in this work on the labyrinth of Bufo vulgaris japonicus larva as to the morphologic study of its perilymphatic spaces, in connection with the membranous labyrinth and auditory capsule, has made special efforts in selecting materials, preparing serial sections, and reconstructing wax models.

The author here wishes to express heartiest thanks to Prof. Dr. T. Asai, director of the institute, for his kind instruction and encouragement.

MATERIAL AND METHODS

The larvae of Bufo vulgaris japonicus were carefully collected for this study, in order to observe the development of the labyrinth during the different stages of the metamorphosis.

In the suburbs of Nagoya the toad usually spawns in the latter part of March. The author captured several parent toads just before the season and kept them in a small aquarium of the institute. The twenty-four-hour-old spawn of a long cord were divided in several bowls with tap-water and placed in the laboratory by a southern window. The water was changed regularly once a day, and water plants were added after the larva, reaching about 12 mm. long, commenced its activity.

The larva of the toad, or tadpole, grows to different sizes, according to its condition of life, both natural and cultural, and, even if similar conditions are maintained, individual differences are scarcely avoidable. The number of larvae were collected at various different stages of development until the end of metamorphosis. The total and corporal lengths of the larvae were measured before the fixation, while the different diameters of the membranous labyrinth were taken on the slides (figs. 31, 32). The fixation was done by Zenker's, Carnoy's, or Orth's solution. The embedding was performed by the celloidin-paraffin combined method. The specimens were made in 10 μ-thick serial sections, and were stained mostly with hematoxylin and eosin, and partly with Best's carmin. For the general study, the hematoxylin-eosin staining was used.

The labyrinth of the Bufo vulgaris japonicus larva is as well developed and as complicated as that of the adult toad. For the purpose of the morphologic study of the labyrinth, a forty-five-day-old specimen with a total length of 24 mm. and a corporal length of 9 mm. was chosen, as this would represent the completed labyrinth of the larva. The membranous labyrinth of this material was 1.03 mm. long, 0.9 mm. wide, and 0.8 mm. high. This material selected represents the organ of the larva during the latter two-thirds of its metamorphic stage, when there is only slightest change in the anatomic and morphologic relations between the membranous labyrinth, auditory capsule, and perilymphatic space.

The wax models of the membranous labyrinth, auditory capsule, and perilymphatic spaces, especially the ductus perilymphaticus, were separately built in the same scale, of which photographs are shown in plates 8 and 9. In this work Leitz's photomicrographic apparatus was used. The technique of reconstruction was Peter's method modified by Prof. Dr. M. Inouye, of Tokyo, whose personal instruction was kindly given to the author.

OBSERVATIONS

A brief history of the development of the labyrinth will be mentioned here. The labyrinth in the beginning, very early in the larval stage, appears as an inlet of thickened ectoderm, which later becomes a closed sac. The sac has a small projection called the recessus labyrinthi which primarily opened outside. This later forms the ductus and saccus endolymphaticus. The main sac develops rapidly at the larval side near the root of the sac (total length of larva, 15 to 16 mm.). Two projections from the dorsal and ventral walls of the sac, meeting each other, make the septum for the canalis semicircularis lateralis (t.l.,[1] 16 mm.). Later, in the same manner, the lateral and cranial walls each separate from the sac at two points, first the canalis semicircularis anterius (t.l., 19 mm.), then the posterius (t.l., 20 mm.). The main sac soon shows a tendency to divide into two sacs in the middle part, just as the semicircular canals were formed (t.l., 20 mm.). The lower portion develops rather slowly, and extends toward the ventral side, especially after the formation of the semicircular canals, which apparently grow from the main sac instead of the upper portion. The lower portion, or pars inferior, in accordance with the development of chondrification of the auditory capsule which has already started (t.l., 20 mm.), will protrude posteriorly to form the lagena and pars basilaris and cranioposteriorly to form the pars neglecta.

The membranous labyrinth, in the beginning of chondrification of the auditory capsule, is enclosed by the embryonic

[1] t.l. = total length.

tissue which gradually forms the periost of the capsular wall and the epithelium of the membranous labyrinth, as well as the so-called perilymphatic tissue. As soon as the chondrification of the capsule progresses, the membranous labyrinth may be observed in its eccentric position against the capsular wall, leaving the perilymphatic spaces at its concave part and around the septal regions.

The chondrification of the auditory capsule starts from the lateral and ventral walls and finishes at the septa. The operculum finally develops independently.

The perilymphatic space, which exists between the auditory capsule and membranous labyrinth, is merely observed as the space filled with embryonic tissue after chondrification, while in the beginning of metamorphosis the saccus endolymphaticus near the bottom of the cranial wall is found (thirtieth day: t.l., 23.5 mm.; c.l.,[2] 8.5 mm.). As the result of liquefaction of embryonic tissue in the space, the perilymphatic fluid and tissue come into existence. A canal projecting from the saccus perilymphaticus extends inside of the auditory capsule, where it develops rapidly. Thus, on the forty-second day (t.l., 24 mm.; c.l., 9 mm.), the entire perilymphatic space is partly occupied by the canal system filled with the perilymph and partly by the meshwork of perilymphatic tissue and fluid.

The ductus perilymphaticus et diverticulae starts its development about the beginning of metamorphosis, and completes it during the first third of the period of metamorphosis, together with the chondrification of the auditory capsule and the extended formation of the membranous labyrinth.

Thus the complicated labyrinth of the larva ceases its morphologic change and development by the end of the first third of the period of metamorphosis, except for a gradual increase in size. At this time the posterior limbs of the larva have already appeared, and the labyrinth will remain in this anatomical condition until the end of metamorphosis.

The labyrinth of the Bufo vulgaris japonicus larva, of which the development was briefly outlined above, will here

[2] c.l. = corporal length.

be studied especially in reference to the morphology of the perilymphatic space in connection with the membranous labyrinth and auditory capsule, during the completed stage which dominates the latter two-thirds of the period of metamorphosis. A separate description will follow of the membranous labyrinth, auditory capsule, and perilymphatic space.

A. Labyrinthus membranaceus

The membranous labyrinth consists of a sac, three canals, and a prolonged duct attachment. Although the sac looks single, it is divided into two parts, the upper and the lower, with an isthmian connection. The upper or pars superior contains the utriculus, to which all three canals are attached. The lower or pars inferior contains the sacculus, to which the three slightly distinguishable portions, the pars neglecta, the pars basilaris, and the pars lagena, are attached, and besides a narrow tubule, the ductus endolymphaticus, which ends in the meningeal cavity as the saccus endolymphaticus.

The utriculus is like an irregularly shaped potato. Its major axis runs anteroposteriorly; the anterior half, obliquely and in anteroventral direction; the posterior, on the contrary, posteroventrally. The upper portion of the utriculus, slightly posterior to the center, where the two vertical semicircular canals join together, is enlarged upward and is called the recessus utriculi superior. The swollen part of the utriculus, which lies cranio-anteriorly at the bottom near the connection with the sacculus, is called the recessus utriculi.

The greater portion of the cranial wall of the utriculus along its major axis and of the recessus utriculi superior has developed eccentrically to form an arch, anchoring to the inner side of the cranial wall of the auditory capsule. The base and lateral side, which adjoin the perilymphatic spaces, are indirectly fastened for the most part to the inner side of the lateral wall of the capsule, together with more or less of the perilymphatic tissue.

The anterior end joins with the ampulla semicircularis anterius from where the canalis semicircularis anterius runs

upward and cranioposteriorly, forming an arch, and finally, ending as a crus simplex at the anterior portion of the recessus utriculi superior. The posterior end joins the ampulla semicircularis posterius. At the anterolateral part of the recessus utriculi the ampulla semicircularis lateralis sends out a conspicuous lateral branch, the canalis semicircularis lateralis, which runs first cranioposteriorly, then laterodorsally, and later horizontally and more posterodorsally, ending at the lateral side of the central portion of the utriculus as a crus simplex.

The canales semicirculares anterius, posterius, and lateralis meet at right angles to each other. The anterius and posterius extend vertically; the lateralis, horizontally. For this reason, the former two are sometimes called the canales verticales, and the latter, the canalis horizontalis. Of all three, the lateral canal is the most developed in size. The ampulla lateralis, situated at the anterior end of the canalis, is the largest of all three ampullae. The canalis semicircularis anterius is smaller than the lateralis, and is the least curved of all. The canalis semicircularis posterius, the shortest of all, has almost the same diameter as the anterius.

The canalis semicircularis anterius, on the middle of its upper side, along its eccentric surface has rather close contact with the inner parts of the auditory capsule. The canalis lateralis touches the capsule comparatively less along its lateroventral side, until it curves anterocranially. All three ampullae adjoin the capsule at their bases.

The anterior half of the pars inferior is occupied by the sacculus, while the lagena and pars basilaris are found in the posterior half together with the pars neglecta, which lies in the craniodorsal corner. The entire sac is nearly oval and the different portions are hardly distinguishable. From the anterior corner of the pars neglecta the ductus endolymphaticus arises and runs upward along the cranial side of the utriculus until it enters the meningeal cavity through the foramen endolymphaticum of the auditory capsule, near the anteroventral corner of the recessus utriculi superior.

The major axes of both the pars inferior and the utriculus run anteroposteriorly and are almost parallel. Posterior to the sacculus, the development of the pars inferior is terminated by the lagena, the pars basilaris, and the pars neglecta.

The lateral walls of the anterior parts of the sacculus and lagena are less convex than the cranial. The anterior parts of these two structures float on the spatium sacculare perilymphatici, and are attached at their bases to the auditory capsule along the major axis as well as the cranial wall of the pars neglecta. The posterior surfaces of the ventral and cranial walls of the pars neglecta face a branch duct of the perilymphatic system, while the rest is almost entirely surrounded by the perilymphatic tissue.

The walls of the sacculus, par neglecta, and pars basilaris, which face the spatium or cavum perilymphaticum, as mentioned above, are very thin and are close to the endothelium of the latter. These parts correspond to Harrison's so-called 'tympanal areas.'

The dorsal portion of the sacculus is funnel-shaped and convex at the center, where the sacculus is connected with the laterodorsal portion of the utriculus through the foramen utriculo-sacculare.

The oval-shaped lagena, which occupies the ventroposterior portion of the pars inferior, develops more cranioposteriorly than the sacculus.

The pars basilaris may be viewed as a laterally swollen part of the dorsal portion of the lagena. Its posterior half is in contact with the recessus partis basilaris perilymphatici, while the rest is surrounded by the greatly thickened perilymphatic tissue.

The pars neglecta is a fairly small, flat attachment to the craniodorsal portion of the sacculus. It extends posteriorly, and its ventral wall is in contact with the perilymphatic system, which is here called the recessus partis neglectae perilymphatici.

B. Capsula auditiva

During the larval period, the auditory capsule is still in the cartilaginous stage. At a later period it will form the labyrinthus osseum by ossification. The capsule consists of the cartilago proto-occipitalis, of which the major anterior portion will later form the pro-oticum and the minor posterior portion, the occipitale laterale.

External view. The auditory capsule from the outside looks as if two tetrahedra were placed together base to base, or, more roughly, like a tiny Brazil nut. One of the three surfaces, facing to the meningeal cavity, corresponds to that of the medial or cranial wall. Another forms the floor of the capsule, running approximately in a horizontal direction. The remaining, or lateral, wall runs obliquely from a craniodorsal direction to a lateroventral.

The anterior minor half of the upper edge of the cranial wall is separated from the lateral wall by the prominentia semicircularis anterius, while the major half is connected with the frontoparietal. The lower edge is connected with the basal wall at its anterior minor half, and is with the parasphenoid, which forms the base of the posterior half of the skull, which is greater. The parasphenoid separates from it, however, and descends along the auditory capsule, later joining the base of the capsule. The foramen jugulare lies between the parasphenoid and the capsule. The central part of the cranial wall, along its major axis, projects upward, following the contour of the cranial wall of the membranous labyrinth inside of the capsule.

The small ostium situated slightly anterodorsally from the center is the foramen endolymphaticum. The two ostia near the middle of the lower edge are the foramina acusticus anterius and posterius, respectively. The two vertical oval openings, near the posterior end, situated considerably above them and parallel to each other, are the foramina perilymphaticum superius and inferius, respectively (fig. 28).

The basal wall joins the lateral wall all along its lateral edge, the prominentia semicircularis lateralis. The cranial

edge directly connects with the cranial wall at the anterior portion, and with the parasphenoid at the posterior portion, which is greater. Near the center, slightly cranial in the posterior half, there is the large oval-shaped fenestra vestibuli, the greater part of which is closed by the operculum. The parts of the foramen cranially situated are developed ventrally. Inside the capsule, the pars inferior of the membranous labyrinth is located in the fenestra vestibuli.

The smaller half of the upper edge of the lateral wall joins the cranial wall at the prominentia semicircularis anterius, and the rest chiefly joins the frontoparietal. The upheaved portion running from near the middle of the upper edge in a ventroposterior direction is the prominentia semicircularis posterius. The lower edge, where both the lateral and the basal wall come together, is called the prominentia semicircularis lateralis.

Internal view. The cavity which is surrounded by the walls of the auditory capsule is called the cavum labyrinthi. The surface of the interior wall of the capsule is more complicated than that of the external. The cavum labyrinthi is divided into the cavum vestibuli communae and the three cava semicircularia. The foramen occupies the greater part of the center, and the latter, the three tunnels which contain the three semicircular canals (figs. 23, 24).

The cavum vestibuli communae contains the main body of the membranous labyrinth, including the utriculus and sacculus with its attached portions. Gaupp has divided this into four parts as the pars superior, pars inferior, recessus anterior, and recessus posterior. The pars inferior is subdivided into the main cavity and the recessus superior. The author, however, here divides the cavum into four parts: the pars superior, inferior, anterior, and posterior.

The pars superior is the part which contains the utriculus and the ductus endolymphaticus; the pars inferior, a shallow inlet, situated below the pars superior, includes the sacculus and its attachments, that is to say, the pars inferior of the membranous labyrinth and the spatium sacculare, recessus

partis neglectae, and recessus partis basilaris of the perilymphatic system; the pars anterior includes the anterior lower portion of the canalis semicircularis anterius together with its ampulla; the pars posterior includes the posterior portion of the canalis semicircularis posterius with its ampulla.

The cava semicircularia are three in number, viz., the cavum semicircularis anterius, lateralis, and posterius. The cavum anterius is the long, narrow tunnel which contains the canalis semicircularis anterius. At its upper end it communicates with the pars superior (recessus superior, Gaupp) of the cavum vestibuli communae through the ostium posterius cavi semicircularis anterioris and at its lower end with the pars anterior of the main cavity through the ostium anterius cavi semicircularis anterioris. The cavum is separated from the cavum vestibuli communae by the septem semicircularis anterius of the auditory capsule.

The cavum posterius also communicates with the pars superior of the cavum vestibuli communae through the ostium anterius cavi semicircularis posterioris and with the pars posterior of the same cavity through the ostium posterius cavi semicircularis posterioris. The cavum is only a short tunnel, separated from the cavum vestibuli communae by a small bridge of the auditory capsule, the septum semicircularis posterius.

The cavum lateralis is situated on the lateral side of the cavum vestibuli communae, from which it is separated by the septum semicircularis lateralis. The anterior end communicates with the pars anterior of the main cavity through the ostium anterius cavi semicircularis lateralis and the posterior end with the pars superior of the same through the ostium posterius cavi semicircularis lateralis. Of all three cava semicircularia, the lateralis is the largest.

Of the three septa semicircularia, the anterius is the most developed; the lateralis, slightly less so; and the posterius is far the least developed.

The outer walls of the cava semicircularia form part of the auditory capsule, where their walls are comparatively thin, while in the neighborhoods of the septa they are fairly thick.

The cavum labyrinthi has six outlets, as previously mentioned; viz., the fenestra vestibuli, foramen endolymphaticum, foramen acusticus anterius and posterius, and foramen perilymphaticum superius and inferius.

C. Spatium perilymphaticum

The spatium perilymphaticum, or perilymphatic space, is a general term given to the spaces existing between the auditory capsule and the membranous labyrinth. The space is partly occupied by a fluid called the perilymph and partly by a network of fiber tissue permeated by the perilymph called the perilymphatic tissue. According to the anatomic and physiologic differences between the two, the author has called the space filled by the fluid the 'ductus perilymphaticus et diverticula,' and the space filled by the network, which merely supports the membranous labyrinth on the walls inside the auditory capsule, the 'pars spongiosa spati perilymphatici.'

1. Ductus perilymphaticus et diverticula. The ductus perilymphaticus et diverticula is a complicated canal system, occupying a considerable area of the so-called perilymphatic spaces. It is situated chiefly around the membranous labyrinth, extending into the meningeal cavity. The system, according to its anatomic relations with other organs, is divided into the spatium sacculare, recessus partis basilaris, recessus partis neglectae, saccus perilymphaticus, and the ductus perilymphaticus, which is the main canal route connecting the other parts. The ductus fenestra vestibuli, first described by Retzius, or the recessus partis vestibuli (Harrison), is entirely indistinguishable (fig. 29).

The spatium sacculare, the largest of all perilymphatic cava within the auditory capsule, runs between the lateral wall of the saccus and the fenestra vestibuli, extending greatly longitudinally and anteroposteriorly. The anterior end of the

spatium touches the recessus utriculi; the posterior end touches the middle part of the lagena and the anterior end of the pars basilaris. The lateral wall of the sacculus, where it comes into close contact with the spatium sacculare, is very thin and slightly convex on the cranial side. On the contrary, the walls of the lagena and pars basilaris are not thin nor especially convex. The spatium sacculare sends out a duct anterodorsally at its anterodorsal corner along the laterodorsal edge of the pars superior of the sacculus. This duct forms the pars communicans of the ductus perilymphaticus (figs. 11 to 15).

Hence the ductus perilymphaticus proceeds almost as far as the cranial wall of the ampulla lateralis, then to the dorsal side of the recessus utriculi, then craniodorsally, later making a sharp curve posteriorly, and finally descending laterоventrally, where the dorsal side of the ductus comes in contact with the lateral edge of the utriculus and the ventrolateral side of the crus simplex semicircularis anterioris. The ductus, descending still farther along the lateral wall of the utriculus to the ostium of the crus simplex semicircularis lateralis, and gradually turning its course from the lateroventral side of the crus to the ventral side, proceeds medially between the crus and the pars basilaris (figs. 11 to 14).

At the ventroposterior wall of the pars neglecta, the ductus has a short projection anterocranially. This is the recessus partis neglectae (Harrison). Because of the thinness of the wall where the ductus comes in contact with the pars neglecta, Harrison has described it as the tympanal area (fig. 14).

The ductus soon enters the cavum cranium through the foramen perilymphaticum superius, which is situated craniposteriorly to the recessus partis neglectae of the auditory capsule (fig. 17).

The ductus gradually enlarges posteriorly along the surface of the capsule, until its lateroposterior end reaches the foramen jugulare. This three-surfaced rhombic portion of the perilymphatic system forms the saccus perilymphaticus (fig. 19). The small portion of the ductus between the

foramen perilymphaticum superius and the saccus may be called the pars meningeale (spatium meningeale, Harrison) (fig. 19).

The saccus perilymphaticus is situated at the corner of the cranial base near the foramen jugulare. One of the three walls of the saccus which encloses the foramen jugulare is the so-called membrana tympani secundaria. The edge is covered with the periost which extends to the occipitale laterale. The saccus has another communication with the inside of the auditory capsule through the foramen perilymphaticum inferius, near the center, slightly anterior to its lateral side. This branch duct enlarges while in the capsule, ending cranioanteriorly at the pars basilaris, with which it comes into close contact. The duct is called the recessus partis basilaris (Harrison), and the portion connecting the saccus with the recessus through the foramen perilymphaticum inferius corresponds to the ductus reuniens named by Harrison (fig. 17).

Thus the perilymphaticus et diverticula is a winding canal system with several enlarged portions. The greater portion is situated within the auditory capsule, and some within the cranial cavity. The system already described may be simply classified as two sacs, the spatium sacculare and saccus perilymphaticus; and two ducts, the ductus perilymphaticus or pars communicans (Gaupp), connecting these two sacs, and a short branch duct or recessus partis basilaris. Going from the spatium sacculare, the main ductus may be divided into the pars ascendens, pars descendens, pars horizontalis, and, outside the capsule, the pars meningeale. The recessus partis neglectae is a short projection from the pars horizontalis, and the recessus partis basilaris has a short neck or the ductus reuniens (Harrison) to join the saccus perilymphaticus (fig. 29).

The wall of the perilymphatic system, especially at the spatium sacculare, pars ascendens and descendens, consists of thin fiber tissue, without the endothelium, while the wall at the pars horizontalis, the recessus partis neglectae, and the recessus partis basilaris is rather thicker than that of the

semicircular canals, with the endothelium of flattened cutaneous cells. The perilymphatic system within the meningeal cavity lacks the endothelium.

2. Pars spongiosa spati perilymphatici. The pars spongiosa spati perilymphatici, which occupies the rest of the part generally called the spatium perilymphaticum (except the ductus perilymphaticus et diverticula), is more or less filled by the meshwork of the perilymphatic tissue. Its density is greatly unequal in different parts of the spatium; the larger part around the membranous labyrinth and ductus perilymphaticus is a honey-combed structure filled by the perilymph, while the so-called connective tissue occupies the parts where the membranous labyrinth comes into closer contact with the capsule. The perilymphatic tissue is the rudimentary fiber tissue of embryologic origin, but its histology may not be discussed here.

The anatomical relations with the membranous labyrinth and the auditory capsule are so complicated that a clear description is very difficult. The physiologic function of the pars spongiosa appears simply to consist in anchoring the membranous labyrinth to the capsular wall. It acts as a safety zone and safeguard for both the membranous labyrinth and ductus perilymphaticus et diverticula in the transmission of vibrations. The perilymphatic tissue is especially thick around the routes of the auditory nerves and contains numerous pigment cells.

The wax model (fig. 30) shows the larger half of the posterior portion of the pars spongiosa, seen from outside. Unnecessary description may be avoided here, as the figures (figs. 8 to 19) will give a better explanation. The location of the pars spongiosa may be described here according to the portions divided into five transverse sections of the labyrinth.

In the first and last of the five sections the pars spongiosa is developed on both the cranial and lateral sides. The lateral side contains more perilymphatic tissue than the cranial, together with the nerves to the cristae (figs. 8, 17, 18, 19).

In the second fifth the membranous labyrinth is fastened at its craniodorsal corner, thus leaving the pars spongiosa on its lateral, ventral, and cranioventral sides. The most anterior part of the ductus perilymphaticus appears near the center of the section above the edge of the vestibulum labyrinthi (figs. 9, 10).

In the central portion of the third and fourth fifths the pars spongiosa is well developed vertically along the anteroposterior axis, because of the eccentric position of the membranous labyrinth on the inner side of the auditory capsule. The greater portion is occupied by the ductus and its diverticula, leaving the pars spongiosa at the dorsal, craniodorsal, cranioventral, and central portions, and all around the semicircular canal except its lateroventral edge, besides the lateral side of the septum semicircularis anterius. The cranioventral portion near and below the two foramina acusticae is packed with the perilymphatic tissue. In the central portion at the height of the foramen utriculo-sacculare, the pars spongiosa extends horizontally for a considerable distance, separating the spatium sacculare from the ductus perilymphaticus. Its anterior end reaches the turning point of the ductus, and the posterior forms the wall between the pars basilaris and the pars horizontalis of the ductus, finally covering the posterior end of the pars inferior of the membranous labyrinth (figs. 11 to 16).

SUMMARY AND CONCLUSIONS

1. While the development of the spatium perilymphaticum starts with the chondrification of the auditory capsule (twentieth day: t.l., 19 mm.; c.l., 6.5 mm.), it exhibits the most distinct formation of the ductus perilymphaticus when the posterior limbs of the larva are half-grown (thirty-fifth day: t.l., 24 mm.; c.l., 8.5 mm.), shortly after the beginning of metamorphosis (fig. 31).

2. The spatium perilymphaticum rapidly completes its development by the end of the first third of the metamorphosis, when the larva reaches its maximum both in total and corporal length (forty-second day: t.l., 24 mm.; c.l., 9 mm.). At this stage it is partly occupied by the perilymph alone, and partly by perilymphatic tissue as well. So that the entire spatium perilymphaticum may be summarized as consisting of the ductus perilymphaticus et diverticula and the pars spongiosa spati perilymphatici (fig. 31).

3. The ductus perilymphaticus et diverticula, beginning its development from the saccus perilymphaticus in the cranial cavity which first extends a duct into the auditory capsule at the beginning of metamorphosis (thirtieth day: t.l., 23.5 mm.; c.l., 8.5 mm.), may be divided into the spatium sacculare, recessus partis neglectae, saccus perilymphaticus, ductus perilymphaticus, and recessus partis basilaris (fig. 6).

4. The ductus perilymphaticus is like a canal which connects two lakes; one in the auditory capsule is the spatium sacculare and the other the saccus perilymphaticus in the cranial cavity. The ductus takes a winding course around the membranous labyrinth within the auditory capsule, sending out a small projection, the recessus partis neglectae, and joins the saccus perilymphaticus after entering the cranial cavity through the foramen perilymphaticum superius. The recessus partis basilaris is a rather short duct which extends independently from the saccus perilymphaticus into the auditory capsule through the foramen perilymphaticum inferius (fig. 6).

5. The ductus fenestra vestibuli, discovered by Retzius and also mentioned by Hane, and the spatium lagenae, first described by Hane, are not found in the perilymphatic system of the Bufo vulgaris japonicus larva before the end of the metamorphosis. The ductus reuniens mentioned by Harrison is still short and exists only in the foramen perilymphaticum inferius (fig. 6).

6. The diverticula of the perilymphatic system, as the spatium sacculare, recessus partis neglectae, recessus partis basilaris, have close contact with the anatomically corresponding parts of the membranous labyrinth. The tympanal areas, as they are called by Harrison, may be connected with certain physiologic functions, such as hearing.

7. The ductus perilymphaticus et diverticulae may play a great rôle in carrying out the functions of both equilibrium and audition. The vibrations may be transmitted through the ductus from the fenestra vestibuli to certain places such as the tympanal areas, where the endolymph of the membranous labyrinth will naturally be affected. The vibrations may then be forwarded by the endolymph to the nerve peripheries of the maculae and cristae. The functions of equilibrium and audition may be in operation, as soon as the rapid development of the ductus system is well advanced, in the early stage of metamorphosis.

8. The pars spongiosa spati perilymphatici, which also developed with the formation of the ductus system and the membranous labyrinth, undergoes no special change nor development before the metamorphosis beyond the attenuation of the perilymphatic tissue. Although the pars spongiosa may not be of prime importance in transmitting vibrations, it serves not only to fasten the membranous labyrinth to the capsular wall, but to act as a safety zone and a safeguard for the functions of both the membranous labyrinth and the ductus perilymphaticus et diverticula.

9. As to the anatomic and morphologic relations of the membranous labyrinth, auditory capsule, and spatium perilymphaticum, I may briefly quote as follows: *a*) If the laby-

rinth be cut transversely into five sections, in the first and last, much like each other, the membranous labyrinth is attached to the capsular wall on its laterodorsal and craniventral edges, leaving the pars spongiosa spati perilymphatici on both the cranial and lateral sides (fig. 3). *b*) In the second of five sections the vestibulum and semicircular canals are fastened only on their craniodorsal edges, thus leaving the rest of the surrounding space to be occupied by the pars spongiosa. In the posterior half of this section the ductus perilymphaticus appears in the central portion (fig. 4). *c*) In the third and fourth of the five sections the vestibulum adheres by its dorsal, cranial, and ventral edges, and the horizontal canal by its lateroventral edge. The remaining spaces between the membranous labyrinth and the auditory capsule are occupied partly by the pars spongiosa, and partly by the ductus perilymphaticus et diverticula at the median portion longitudinally in the anterior half of this section, and the same horizontally in the posterior half (fig. 5).

10. The ductus perilymphaticus et diverticula may have more important physiologic relations than the membranous labyrinth in connection with the cranial cavity and spinal cord, as to the change of pressure, the transmission of vibrations, the osmosis of fluid, etc., because of the freer contact between the former and the cranial cavity at the saccus perilymphaticus, while the latter with the long, narrow ductus endolymphaticus enters the cranial cavity, ending as the saccus endolymphaticus (fig. 6).

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PLATES

ABBREVIATIONS

(Capital letters are used only for parts of the perilymphatic system.)

aa, ampulla semicircularis anterius
al, ampulla semicircularis lateralis
ap, ampulla semicircularis posterius
ca, crista acustica anterior
cl, crista acustica lateralis
cn, crista acustica neglectae
cp, crista acustica posterior
csa, canalis semicircularis anterius
csl, canalis semicircularis lateralis
csp, canalis semicircularis posterius
de, ductus endolymphaticus
DP, ductus perilymphaticus
DR, ductus reuniens
faa, foramen acusticus anterius
fap, foramen acusticus posterius
fe, foramen endolymphaticum
fj, foramen jugulare
fv, foramen vestibuli
FPI, foramen perilymphaticum inferius
FPS, foramen perilymphaticum superius
fus, foramen utriculo-sacculare
l, lagena cochleae
ml, macula (papilla) acustica lagenae
mru, macula acustica recessus utriculi
ms, macula acustica sacculare
op, operculum
pb, pars basilaris
pn, pars neglecta
PM, pars meningeale
psa, prominentia semicircularis anterius
psl, prominentia semicircularis lateralis
psp, prominentia semicircularis posterius
RPB, recessus partis basilaris
RPN, recessus partis neglectae
ru, recessus utriculi
s, sacculus
se, saccus endolymphaticus
SP, saccus perilymphaticus
SS, spatium sacculare
ssa, septum semicirculare anterius
ssl, septum semicirculare lateralis
ssp, septum semicirculare posterius
sus, sinus utriculi superior
u, utriculus

PLATE 1

EXPLANATION OF FIGURES

1 to 5 Schemas of transverse sections of the Bufo vulgaris japonicus larvae at different stages, showing the relations between the auditory capsule, the membranous labyrinth, and the perilymphatic space. *a*, capsula auditiva; *b*, labyrinthus membranaceus; *c*, pars spongiosa spati perilymphatici; *d*, ductus perilymphaticus et diverticula.

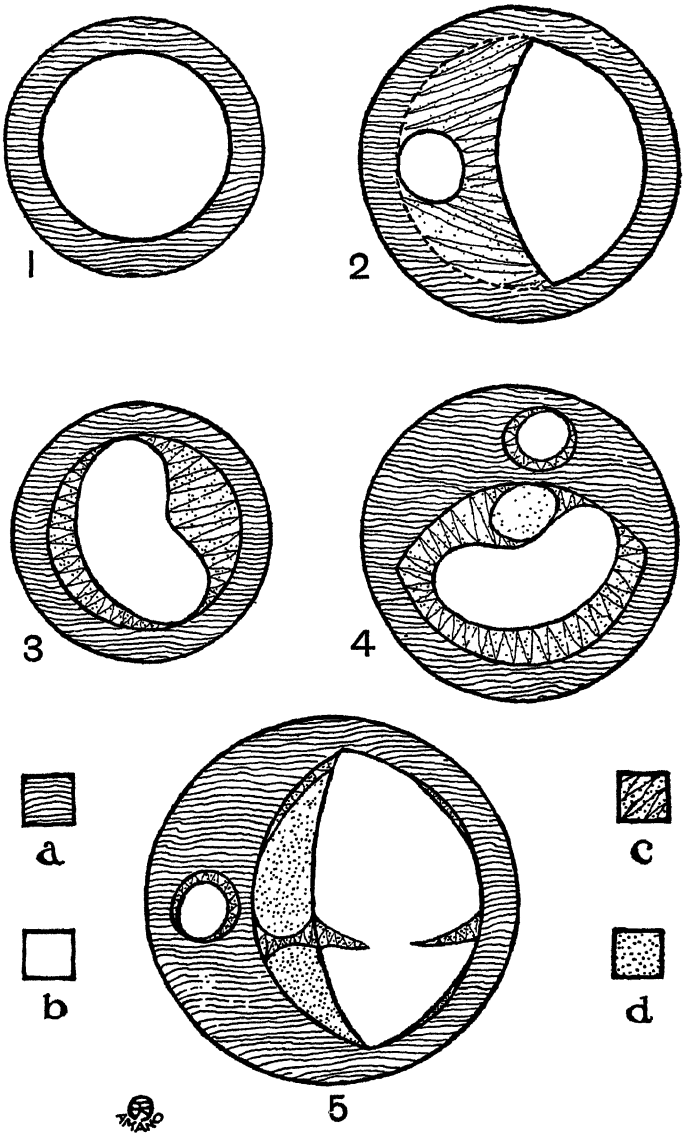
1 A young larva before chondrification of the auditory capsule. The auditory capsule is in the stage of 'Vorknorpel.' No perilymphatic space between the capsule and membranous labyrinth is found.

2 A larva with the membranous labyrinth in the process of further growth, and the auditory capsule in that of chondrification. The septum separating the semicircular canal from the vestibulum is filled with 'Spindelknorpel,' and the liquefaction of the embryonic tissue in the perilymphatic space is now under process.

3 The first and last of five sections of the labyrinth of the larva, during the second and last thirds of the period of metamorphosis. This schema corresponds to figures 8, 17, 18, and 19 of the microscopic sections.

4 The second fifth of the labyrinth of the same specimen as figure 3. The schema corresponds to figures 9 and 10.

5 The third and fourth sections of the same as figures 3 and 4. This schema corresponds to figures 11 to 16.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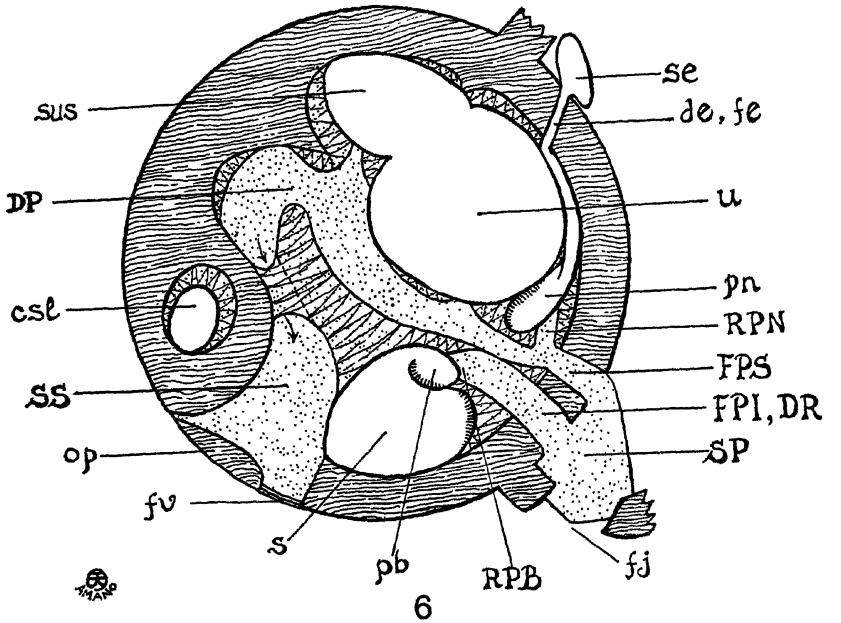
LABYRINTH OF BUFO VULGARIS JAPONICUS

KAGEYAS W. AMANO



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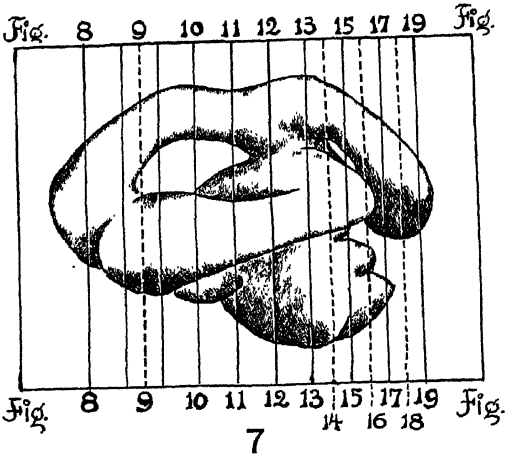
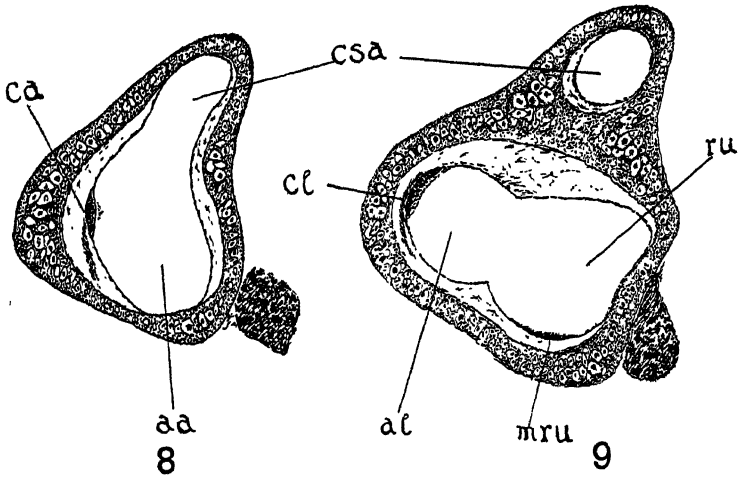
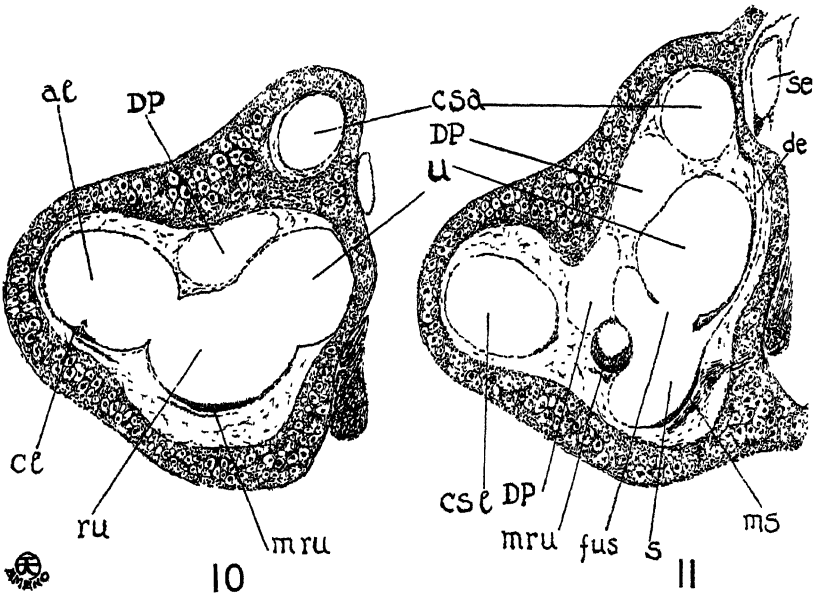


7

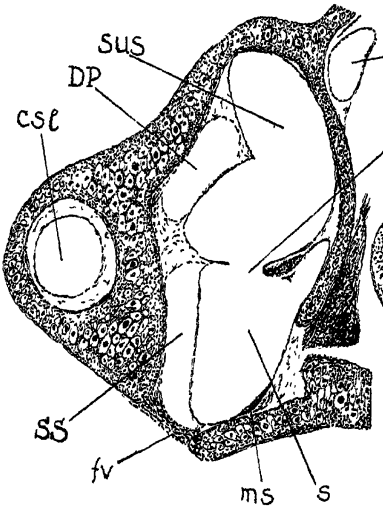
6 Schema of the labyrinth of the larva, during the latter two-thirds part of metamorphosis, made from many sections of different parts, showing especially the relations between the ductus perilymphaticus and the membranous labyrinth.

7 The numbers indicate the following figures, showing the position of the microscopic sections.





8 to 19 Microscopic sections of the labyrinth of a 24-mm. (t.l.) and 9-mm. (c.l.) Bufo vulgaris larva, at intervals of 50 to 100 μ.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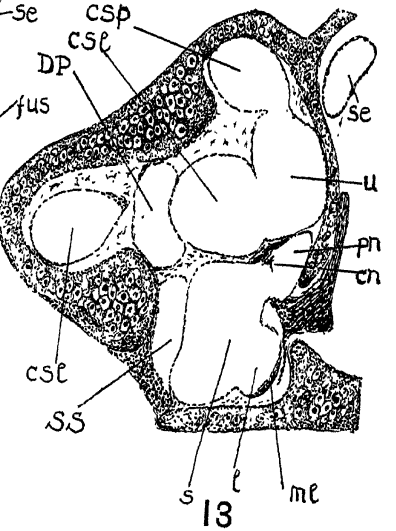
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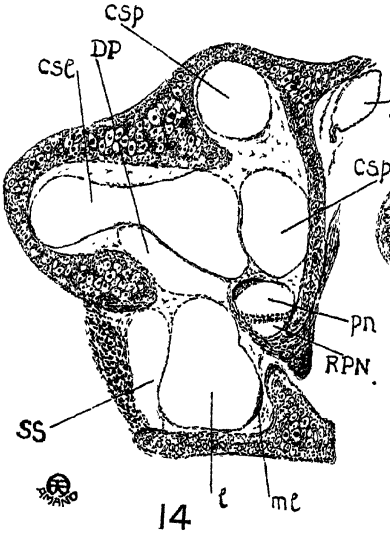
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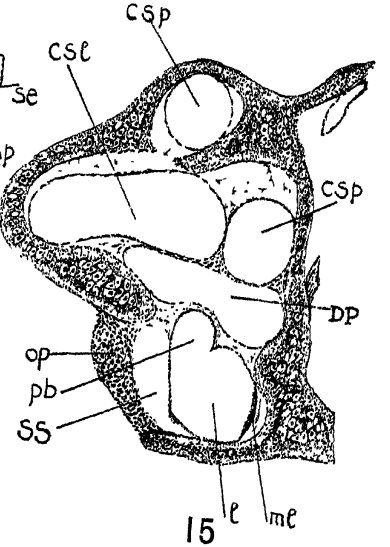
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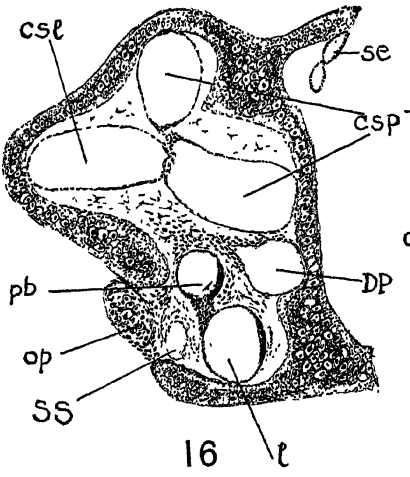

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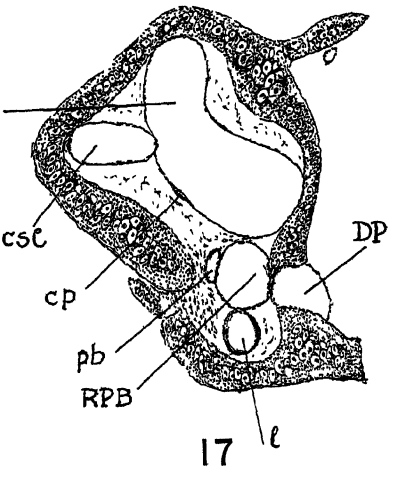

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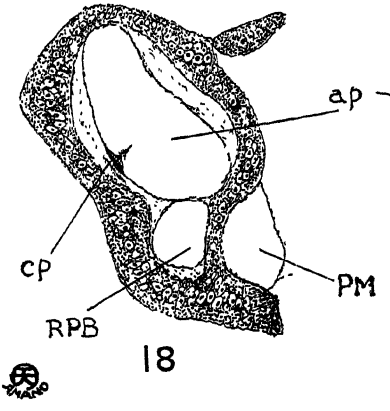

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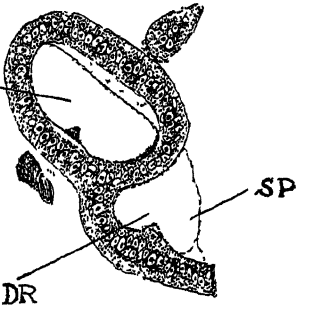

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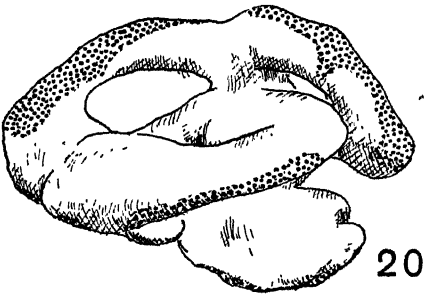
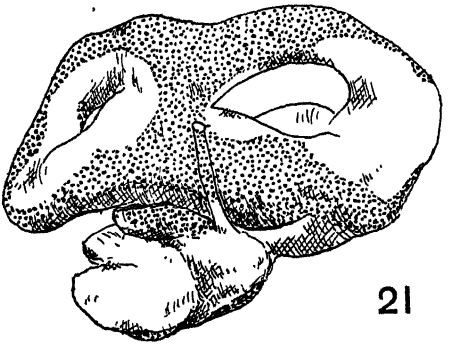
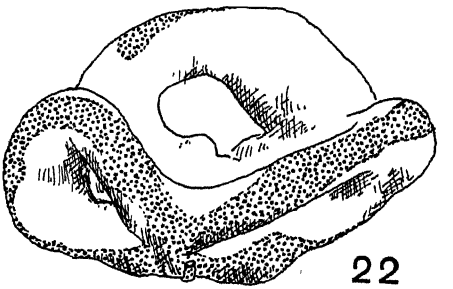
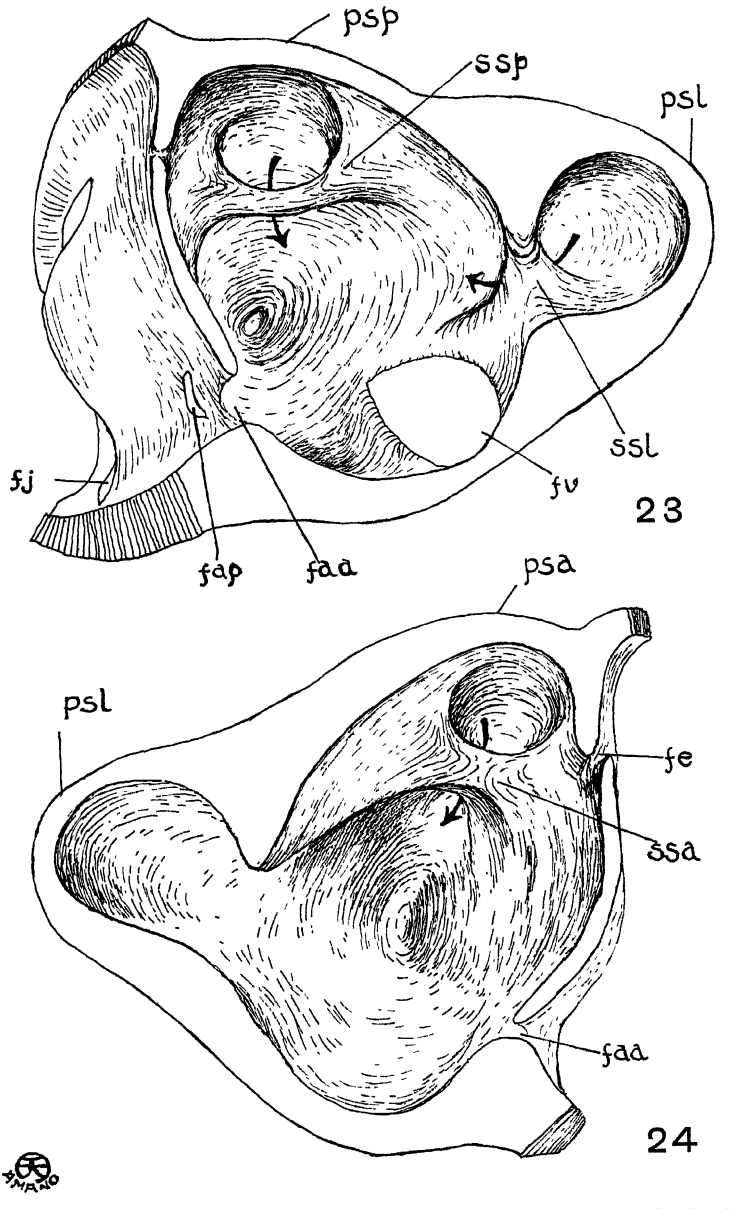


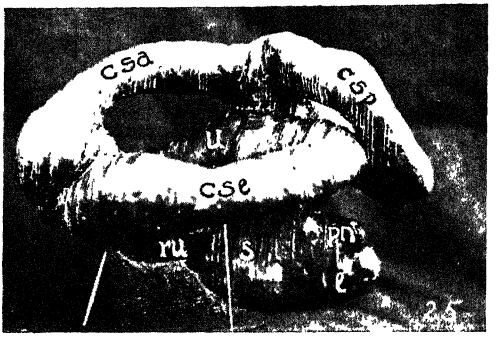


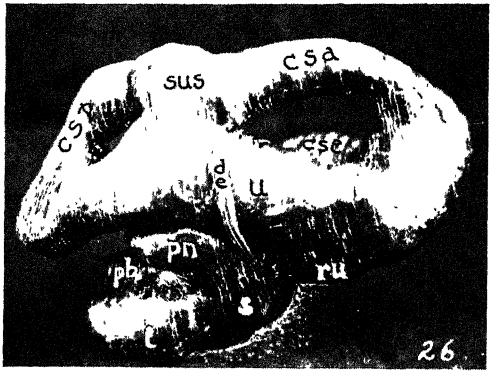


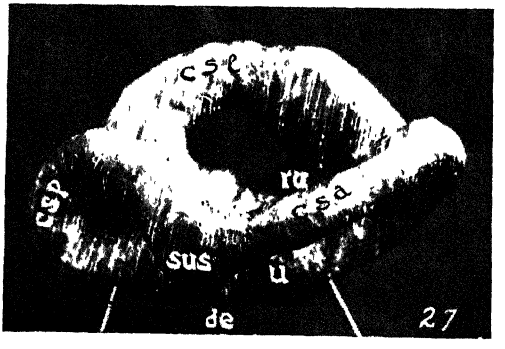
The dotted parts of the membranous labyrinth show where its surface touches the inner wall of the auditory capsule. The other parts are in touch with the perilymphatic space. Figure 20 is seen from the lateral; figure 21, from the cranial, and figure 22, from the dorsal aspect.



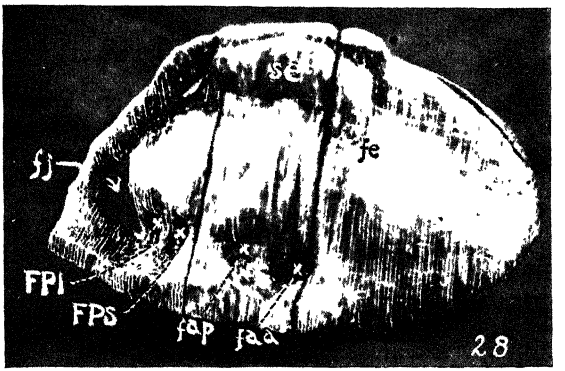
The interior views of the auditory capsule. Figure 23 is the anterior half of the capsule and figure 24, the posterior.

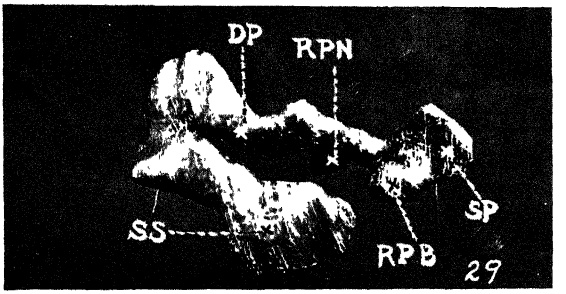


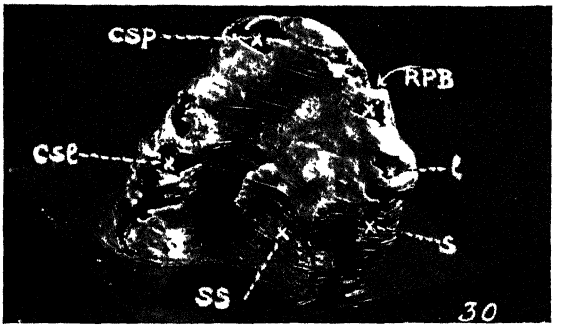




Photographs of a wax model of the membranous labyrinth of a 24-mm. (t.l) and 9-mm. (c.l.) Bufo vulgaris larva. Figure 25 is seen from the lateral; figure 26, from the cranial, and figure 27, from the dorsal aspect.



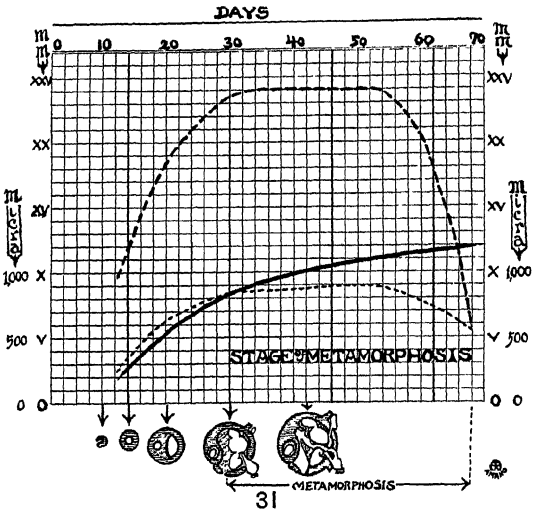




28 Wax model of the auditory capsule of the same specimen as in plate 8, showing its cranial surface, with the saccus endolymphaticus.

29 Ductus perilymphaticus with its diverticula photographed from the latero-dorsal side.

30 The larger posterior half of the pars spongiosa spati perilymphatici, as seen from the posterolateral side. The black areas indicate where the membranous labyrinth is attached to the auditory capsule.



31

31 Chart showing the development of the labyrinth in comparison with the changes in the total and corporal lengths of the larva.

———, total length of larva in millimeters. – – – – –, corporal length of larva in millimeters. ———, anteroposterior diameter of the membranous labyrinth in micra.

	SPECIMEN NO.	AGE	TOTAL LENGTH	CORPORAL LENGTH	MEMBRANOUS LABYRINTH		
					Length	Width	Height
		Days	*Mm.*	*Mm.*	*Micra*	*Micra*	*Micra*
	I	18	16	5.5	400	280	320
	II	20	18	6	500	290	400
	III	21	19	6.5	570	410	430
	IV	22	20	7	650	500	470
	V	25	21	8	700	540	500
	VI	27	22	8.5	800	600	550
Metamorphosis	VII	30	23	8.5	850	650	600
Metamorphosis	VIII	35	24	8.5	900	700	650
Metamorphosis	IX	45	24	9	1030	900	800
Metamorphosis	X	54	24	9	1150	950	850
Metamorphosis	XI	61	20	7	1160	970	880
Metamorphosis	XII	68	6.5	6.5	1200	1000	900

32

32 Table showing the size of the larva, in the total and corporal lengths, together with the size of the membranous labyrinth.

THE SPERMIOGENESIS OF SUCCINEA OVALIS SAY, WITH SPECIAL REFERENCE TO THE COMPONENTS OF THE SPERM

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Department of Biology, De Pauw University

TWO TEXT FIGURES AND EIGHT PLATES (SEVENTY-EIGHT FIGURES)

AUTHOR'S ABSTRACT

An investigation of the spermiogenesis of Succinea ovalis Say, a small terrestrial pulmonate, has revealed. 1) The germ cells are differentiated from indifferent germinal epithelial cells. In this form the germinal epithelium is a true epithelium, and not a syncytium. 2) Forty chromosomes are found in the spermatogonial divisions and twenty in the maturation divisions. 3) Early in spermiogenesis the proximal centriole penetrates through the spermatid nucleus and, with the oxychromatin, forms an intranuclear rod similar to that reported for certain prosobranchs. The homology and significance of the rod are discussed. 4) Of the cytoplasmic structures, the mitochondria and the Golgi apparatus were followed through all stages of spermatogenesis. 5) At the maturation divisions the mitochondria are grouped into peculiar, thread-like structures. Some of the mitochondria take part in the formation of the sheath around the axial filament of the spermatozoon, while the remainder are sloughed off with the cytoplasmic remnant. 6) The Golgi apparatus consists of a number of banana-shaped rods closely grouped around the idiosome. Three to five Golgi rods are found in the spermatid stages. A portion of the Golgi apparatus and idiosome (acroblast) forms the acrosome, and the Golgi remnant is discarded at the end of spermatogenesis. 7) In mature sperm both head and tail have a spiral structure. The origin and nature of the spirals are pointed out.

CONTENTS

Introduction 244
Material and methods 245
A. Material 245
B. Methods 246
a. Fixation 246
Hermaphroditic gland (ovotestis) 246
A. Structure 246
B. Seasonal cycle 247
General features of spermatogenesis 249
A. Nuclear features to the end of the maturation divisions 249
a. Spermatogonial stages 249
b. Primary spermatocyte stages 250
c. Secondary spermatocyte stages 253
B. Cytoplasmic features to the end of the maturation divisions 253
a. Mitochondria 253
b. Golgi apparatus 255
Components of the spermatozoon 257
A. Acrosome 257
B. Intranuclear rod 260
C. Spiral structure 264

Spermatid remnant 266
Chromatoid body 268
Mature sperm 268
Conclusions 270
Literature cited 271

INTRODUCTION

The object of the present study has been to make a careful investigation of the spermiogenesis of a pulmonate gastropod, endeavoring, by the use of the most up-to-date technique for both nuclear and cytoplasmic phenomena, to clear up as far as possible the discrepancies which have arisen from the accounts of previous workers. A great deal of confusion exists regarding the components of pulmonate spermatozoa, particularly the acrosome and the spiral structure. Platner ('85), one of the earliest workers on pulmonates, states that the acrosome arises from one of the centrosomes of the spermatid. Gatenby ('18), a recent worker, thinks that the acrosome originates from a special body which bears no relation to the other elements of the spermatid stage. Prenant ('87) and Kleinart ('09) explain the origin of the acrosome as merely a thickening of the anterior nuclear wall. Recent work in forms other than pulmonates indicates that the Golgi apparatus and idiozome play a prominent part in its formation.

We find the same diversity of opinion regarding the spiral arrangement. Platner, in the paper already mentioned, claims that both the head and tail are spirally twisted. Retzius ('06) believes that the spirals are confined to the tail. Most workers on pulmonates, however, neglect the entire subject or deal with it in a superficial way.

Although an intranuclear rod has been described in the sperm of many forms, the present study represents the first attempt to work out its origin and development in pulmonates. Especial emphasis has been laid upon a comparison of the rod in pulmonates with that found in other forms, and the aim has been to find whether or not there is a common plan of formation.

While the final stages of sperm development have been emphasized here, I have also presented the earlier stages where, of course, many of the sperm elements are first laid down. I have followed the origin and development of the cytoplasmic inclusions and their close relationship to the nuclear elements. Therefore, it seems most logical to present together both cytoplasmic and nuclear phenomena as phases of one general process, the origin and differentiation of the spermatozoon.

I wish to express my most sincere appreciation for the kind help of Prof. Edwin G. Conklin, of Princeton, under whose direction the major part of this work was done, and to thank Prof. Ulric Dahlgren, who so kindly placed at my disposal a room and other facilities at the Harpswell Biological Station, Mount Desert Island, Maine, during the summer of 1923.

MATERIAL AND METHODS

A. Material

Succinea ovalis is a small terrestrial pulmonate very widely distributed over the eastern states and usually abundant wherever found. It occurs in low moist places, on grass and other vegetation in the summer, and under decaying leaves and rubbish during the hibernating season. Part of the material used in the investigation was collected near the Harpswell Biological Laboratory on Mount Desert Island, Maine. The snails were found there along a small temporary brook that ran through a wood not far from the station. This collection was made in the months of June, July, and August. For the other months of the year, the material was obtained in a large swamp near Princeton, New Jersey. Collections were made in every month of the year, in order to have complete all the stages of the seasonal reproductive cycle.

In dissecting out the ovotestis the shell was first split open and quickly removed from the visceral mass. Then, immersing the latter in Ringer's fluid, the ovotestis was separated out from the liver and dropped into the fixing fluid. Usually

some of the liver was cut off and fixed with the ovotestis, as the gland is small and so deeply embedded in the surrounding liver that it is very difficult to separate the two completely. Although the liver offers some hindrance to good fixation, these difficulties may be eliminated by fixing small pieces.

B. Methods

a. Fixation. A number of fixatives were used for both nuclear and cytoplasmic structures. For nuclear structures Flemming strong for forty-eight hours, Bouin for twenty-four hours, and Benda-Flemming for forty-eight hours gave the best results. Bouin's fluid was especially good for chromosomes and centrioles. In the case of cytoplasmic elements Benda-Flemming and Flemming-without-acetic were the most successful. I found that prolonged fixation in the latter intensified the clear appearance of all cytoplasmic bodies. In one case where ovotestes were left in this fluid for three weeks, both mitochondria and Golgi rods were demonstrated with remarkable clearness.

b. Staining. Heidenhain's iron hematoxylin was found to be far the best for general structures. Eosin and orange G were successfully used as counterstains. Special stains, such as Champy-Kull's acid fuchsin, Benda's alizarin-crystal violet, and Bensley-Cowdry acid fuchsin and methyl green, gave fair results for staining cytoplasmic elements. Most of these stains, however, are very fickle and I eventually adopted iron hematoxylin following Flemming-without-acetic, even for the mitochondria and Golgi apparatus.

HERMAPHRODITIC GLAND (OVOTESTIS)

A. Structure

The ovotestis of Succinea is embedded in the liver in the upper whorl nearest the apex. It is a lobed structure of a dull whitish color, and varies in size with different individuals and at different seasons of the year. At the height of activity the gland measures about 3 mm. in length by about half that in thickness. Its general contour conforms to that of the whorl of the shell in which it is found.

The gland is made up of many acini pressed closely together, each opening into an atrium which communicates with the hermaphroditic duct. Around the acini the walls have two well-marked layers. The outer one, of a fibrous nature with small inconspicuous nuclei, is known as Ancel's layer and can scarcely be detected. The inner layer is the true germinal epithelium (fig. 1); it is made up of a row of flattened cells with narrow, compressed nuclei (fig. 4). With proper fixation the cell boundaries appear distinct, and the epithelium is thus not a syncytium. Buresch ('12), on Helix arbustorum, represents the germinal epithelium as being a true syncytium. On the other hand, Gatenby ('17, '18), in Helix aspersa and other forms, shows that the germinal epithelium is a true epithelium, consisting of a row of flattened cells with compressed nuclei very much like those I have found in Succinea.

In the more differentiated germinal epithelium we find, in addition to these indifferent cells, the same elements which Gatenby ('18) has described in a number of pulmonates. These are the nurse, ova, and male cells. Most investigators of pulmonate gastropods are in agreement on the origin of the germ cells from indifferent germinal epithelial cells. Buresch ('12) finds such to be the case in Helix arbustorum, and more recently, Gatenby ('17, '18) states that in Helix aspersa and some other snails the various reproductive elements arise by differentiation from the germinal epithelial cells. The evidence obtained in this study of Succinea confirms these two observers, as the transformation of the indifferent cells into the various germ elements can, with proper technique, be traced.

B. Seasonal cycle

The activity of the gland varies considerably at different periods of the year. By fixing specimens each month, this cycle has been closely followed. About the latter part of September the gland becomes practically inactive and remains so until late in March (in New Jersey). This period of

glandular inactivity extends somewhat beyond the period of hibernation. At no time can the gland be said to be totally inactive, because some dividing cells can nearly always be found. Fixation of the gland, especially in the older snails, is very unsatisfactory at this season because of the large amount of degeneration going on. The acini appear as large empty spaces, with an occasional sex cell in the germinal epithelium and many disintegrating elements in the lumen.

The hibernation of Succinea begins early in October. During the spring and summer their natural habitat is on the vegetation of wet and marshy regions. With the coming of the early frosts they quickly descend to the ground to make their winter home under decaying leaves and rubbish, or even down in the loose soil. They seem remarkably well adapted to the harsh conditions of this environment, for the writer has found specimens in a swamp near Princeton, even after the severe floods and freezes of January and February. These individuals, when taken into the laboratory, quickly became active and seemed no worse for the change.

In early spring, when the snails emerge from hibernation, the ovotestis becomes active, and the development of the germ cells, once started, proceeds with great rapidity. While in specimens fixed on the 21st of March the ovotestes were still dormant, those fixed two days later showed decided indications of the development of both male and female cells. I could find no evidence that growth of the cells of either sex preceded that of the other. Succinea is therefore neither protandrous nor protogynous, but a simultaneous hermaphrodite.

In the glands of hibernating snails are numbers of degenerating male and female cells of various sizes. They are found in all parts of the acini, often obscuring the normal germ cells. There is no evidence that they serve as food for the active germ cells. At this time the germinal epithelium also shows degenerative changes. The disintegrating cells stain deeply in iron hematoxylin, and even a prolonged immersion in iron-alum fails to give a satisfactory differentiation.

The activity of the gland continues throughout the summer months. Mature eggs were found as early as March in New Jersey and as late as the middle of August in Maine, so that the egg-laying period must extend over several months. However, formation of sex cells continues in the ovotestis for an even longer period.

In order to hasten the activity of the ovotestis, snails were placed on ice in refrigerators for varying periods of time. They were then removed to room temperature and left for several weeks. If this arrangement adequately simulated the wintering conditions of the snails, sex cells should begin to form soon after removal from the ice. Experiments showed that snails left on the ice for two weeks or less and then restored to room temperature showed no changes in sex activity. On the other hand, about 40 to 50 per cent of those left on ice for from three to four weeks formed sex elements a few days after their removal, and a few even laid eggs. The mortality among snails so treated, however, was very great. This is hard to explain, since snails frequently undergo in their native habitat even more severe conditions and yet manage to survive and reproduce.

GENERAL FEATURES OF SPERMATOGENESIS

A. Nuclear features to the end of the maturation divisions

a. Spermatogonial stages. Spermatogonia first appear when the indifferent germinal epithelium gives rise to the progerminative cells (fig. 2). This takes place in the following way. The nuclei of the germinal epithelium increase in size and become markedly rounded (fig. 2, *m.p.c.*). At the same time the chromatin clumps, so characteristic of indifferent cells, are broken up into less deeply staining masses. These new cells then increase in size and eventually fall into the lumen to form the primary spermatogonia. These later divide to produce the secondary spermatogonia (fig. 1, *s.sg.*). Both primary and secondary spermatogonia are unusually abundant in the spring, becoming less numerous with the advance of the season.

In size and shape the spermatogonia are readily distinguishable from the spermatocytes. The former are commonly spherical, although less frequently they are oval or polygonal (fig. 3). One of their most striking features, particularly of the secondary spermatogonia, is the small amount of cytoplasm in comparison with the size of the nucleus (figs. 8 and 9). The nuclei are usually quite granular in appearance. The chromatin does not stain deeply, and the reticular network is inconspicuous (figs. 2 and 3).

At the height of the division period, in early spring, the acini contain many examples of the various stages of division (figs. 5, 6, and 7). When a primary spermatogonium begins to divide, the chromatin clumps in the nucleus organize into numerous long, curved, rod-like chromosomes lying so close together that it is quite difficult to ascertain their correct number. But, on the assumption that there are twice as many chromosomes in the spermatogonial divisions as there are in the reduction divisions, there must be forty chromosomes in these early stages. Their characteristic shape makes them easy to distinguish from the chromosomes of the maturation divisions (figs. 5 and 6).

The anaphase and telophase stages follow, and thus the cell is divided into secondary spermatogonia (figs. 8 and 9). The latter are somewhat smaller than the primary spermatogonia, and in the resting condition their nuclei take a darker stain. They also contain a relatively smaller amount of cytoplasm (compare figs. 3 and 8).

The divisions of the spermatogonia follow each other rapidly, and the daughter cells are pushed out into the lumen, where they eventually differentiate by growth into primary spermatocytes (figs. 10 and 11). Although spermatogonia are most numerous in the spring, they are found in mitotic division throughout the season of activity. During the winter months little division takes place.

b. Primary spermatocyte stages. The secondary spermatogonia differentiate into primary spermatocytes through growth and development (fig. 10). Although the latter can-

not be distinguished easily from the spermatogonia, they are, nevertheless, slightly larger and differ also in certain other respects. As a general rule, the relative amount of cytoplasm has increased and has become more vacuolated. In the resting nucleus of the spermatocyte the chromatin is distributed in fine granules connected by a delicate reticulum (fig. 11). The chromatin takes the stain more deeply, and there are usually present one or two nucleoli. Commonly the spermatocytes are grouped together, although occasionally they occur singly in the lumen. A characteristic cluster will reveal a number of them attached to a nurse cell. Material fixed in strong Flemming shows two centrioles in the idiozome (fig. 10). Spermatocytes are especially abundant in early summer, although some are present during most of the gland's activity.

The chromatin of the nucleus breaks up into small structures, and these are arranged in fine bead-like threads which form a loose mass of coils in the nucleus (fig. 12). These are the leptotene threads and they are made up of darkly staining granules connected with narrow, less deeply staining bridges. The threads form such a compact mass that it is difficult to distinguish whether they form independent elements or a continuous thread. In the next stage the threads are oriented toward one pole of the nucleus, forming the leptotene bouquet stage (figs. 13 and 14). In this case the threads have their free ends attached at one pole of the nucleus, thus forming loops. These loops vary in length and are so numerous that they cannot be counted accurately. A still later stage is shown in figure 15, where some of the loops are large and double, while others are smaller and evidently single. This probably indicates that a side-by-side pairing of the threads (parasynapsis) has occurred. These pachytene loops are large, with their free ends oriented toward one pole and with their loops filling up a large part of the nuclear space. The attached ends of the loops are always directed toward the place where the cytoplasm is most abundant and where the idiozome can plainly be seen (fig. 15).

The chromatin loops gradually become shorter and thicker, with peculiar thorn-like growths on their surfaces (fig. 16). There is no trace of a furrow between the original pairs of parallel threads.

The threads finally break up (resolution phase), contract, and condense into paired rods (figs. 17 and 18). Later, a longitudinal split occurs along the line of synaptic union, leaving the ends of each pair still connected (fig. 19). Each pair now forms a ring (figs. 20 and 21) which later condenses into the definitive tetrad. The tetrads are all of the same shape, although some are larger than others (figs. 22 and 23).

After the dissolution of the nuclear wall, the tetrads can be seen on the spindle as bivalent chromosomes (figs. 24 and 25). Polar views of the metaphase plates show twenty of these chromosomes (figs. 26 and 28). The number reported in most other pulmonates is slightly larger. Kleinart ('09) states that the haploid number is twenty-four in both Helix hortensis and H. nemoralis; Murray ('98) gives twenty-four for H. pomatia; and Buresch ('12) finds the same number in H. arbustorum. In Succinea the larger chromosomes appear at the periphery, and most of them show a characteristic dumbbell shape at this stage. This grouping of the chromosomes is quite characteristic of the metaphase plates. They are shorter and thicker than the chromosomes of the spermatogonial divisions (compare figs. 6 and 24).

All stages of the first maturation division can be seen in figures 24 to 33. In the anaphase the chromosomes draw together into a compact mass, forming the daughter-chromosome plate (fig. 29). In this plate the individual chromosomes can rarely be distinguished, but all are crowded together, forming a darkly staining mass (fig. 30). A chromatoid body (fig. 27) is now found in one of the daughter cells (fig. 31). In the later stages of this division the spindle becomes less clear (fig. 32) and eventually leaves only traces or remnants (figs. 33, 34, and 35). A midbody can be made out in some instances, especially after certain fixations, such as Bouin's (fig. 32). It consists of a few darkly staining granules lying directly in line with the constriction of the cell body.

c. Secondary spermatocyte stages. The resting stage of the secondary spermatocyte is of short duration and not easily found (figs. 34 and 35). The compact chromosome plates (fig. 31) loosen up, a nuclear membrane is formed around the chromatin material, and eventually a stage occurs like that shown in figure 33. The chromatin masses of these resting stages probably represent individual chromosomes. A spindle remnant is visible in all resting stages (figs. 34 and 35). As the resting stage is very brief, there quickly follows the beginning of the second maturation division. The smaller size of all the parts distinguishes this division from the first. Side and polar views of the metaphase plates are shown in figures 36, 37, and 38. The chromosomes, it will be noticed, are smaller than those in the first maturation division. The spindle, which is short and thick in the early stages of this division, elongates in the anaphase stages (fig. 39) and decreases in diameter. At the same time the interzonal part of the spindle appears to push the daughter-chromosome groups before it to opposite sides of the daughter cells (fig. 40). The cell constriction is quite completed by this time, although an attachment between the two spermatids persists until later (fig. 42). Groups of these daughter spermatids can often be found, indicating that the union is maintained long after the complete separation of cell bodies. The spindle remnant, usually quite marked, may be the main factor in the attachment.

B. Cytoplasmic features to the end of the maturation divisions

a. Mitochondria. When the nucleus of the indifferent germinal epithelial cell has assumed the oval shape characteristic of the male progerminative cell, a small cluster of granules appears at one side of the nucleus (fig. 2). This aggregation forms a dense, compact mass, so that it is quite difficult to distinguish individual granules. I have succeeded in demonstrating this body only with Flemming-without-acetic as a fixative. My impression is that these granules are

mitochondria, but that they are especially hard to fix at this time. Gatenby ('17) in Helix finds that the mitochondria are much easier of demonstration at certain stages than at others, and infers from this that they may vary in their chemical reactions. When the male progerminative cell falls into the lumen of the acini to become the primary spermatogonium, this cloud of granules breaks up, and the particles are distributed through the cytoplasm (fig. 3). During the spermatogonial divisions they are fairly evenly divided between the daughter cells (fig. 7). At these divisions they do not align themselves into the bead-like rows found later during maturation (compare figs. 7 and 25). The mitochondria stain uniformly dark with iron hematoxylin, although not so black as chromatin material.

During the resting primary spermatocyte stages, the mitochondria remain scattered through the cytoplasm as distinct granules (figs. 10 and 11). There is no tendency toward any particular arrangement. Later, however, about the time of the resolution phase, the granules begin to form into thread-like strands (fig. 18). These threads are short, and usually the granules of which they are composed are rather loosely arranged, indicating that the threads are just forming. A side view of the metaphase in the first maturation division shows the mitochondria definitely arranged into threads so numerous that it has not been possible to count them accurately (figs. 24 and 25). Each thread is short and is made up of from six to ten granules. In preparations fixed with Flemming-without-acetic they show very clearly. In these stages the strands are scattered throughout the cytoplasm, but those in the region of the spindle are parallel to its axis. When the primary spermatocyte divides, the mitochondrial threads are distributed approximately equally between the secondary spermatocytes (fig. 30). During the division the individual threads do not divide, but go intact to the daughter cells. In Helix aspersa Gatenby and Ludford ('21) describe a similar behavior of the mitochondria during the maturation divisions. Their statement that the "mitochondria, which

previously were rounded, have metamorphosed to form rodlets" is not explicit enough to indicate whether the rodlets are formed from single granules or from a combination of granules which cluster together. From their drawings, however, I interpret each rodlet to be made up of several granules.

In the secondary spermatocytes the mitochondria, some of which are still in the form of threads, are scattered through the cytoplasm (figs. 34 and 35). During this brief stage they do not undergo any change. At the time of the second maturation division, the threads are arranged in the cytoplasm in a manner similar to the corresponding stages of the first maturation division. They are found throughout the cytoplasm and also on the spindle during the anaphase stages. At the actual division each spermatid receives about half the total number of threads (fig. 39).

b. Golgi apparatus. The Golgi apparatus also arises during these early stages. Material fixed in Flemming-without-acetic and also in Bowen's osmic-acid fixation reveals certain small, darkly staining elements lying close to the nucleus of the secondary spermatogonium (fig. 9). These are the Golgi rods. They are larger than the mitochondria, and the two are, in my opinion, separate and distinct structures. I have been unable to trace these rods back to an earlier origin. There is nothing apparent in the early male progerminative or primary spermatogonial cells that can be considered as the forerunner of these rods. It was impossible to determine the relationship, so prominent in later stages, of the rods and the idiozome. Neither could the exact number of rods in the secondary spermatogonium be ascertained with certainty.

The Golgi rods in the primary spermatocyte are found grouped around the idiozome (fig. 11). Although commonly they are too closely massed for accurate counting, their number can, in favorable preparations, be determined and varies between fifteen and nineteen. The rods are distinctly banana-shaped and stain uniformly dark. The shape of the

Golgi rods in Succinea is like those described by Gatenby ('18) for Helix, Limax, and other pulmonates.

In the early prophases of the first maturation division the apparatus is still found in a compact mass around the idiozome (fig. 13) and resembles in most respects those of the earlier spermatocytes. After the resolution phase, however, the rods become scattered in the cytoplasm. As the rods move away from each other they evidently bear with them the idiozome material in which they were formerly embedded, for the idiozome is no longer a compact mass (figs. 18 to 22). This is the beginning of a definite separation of the rods, which we find in later stages accumulated in two well-marked masses (fig. 23). Whether there is a centriole in each of these masses could not be determined.

At the metaphase these Golgi rods are collected in a compact mass at each pole of the maturation spindle (fig. 27). Each mass probably contains an approximately equal number of rods. In the division that follows, the rods pass into the two daughter cells, but there has been no division of individual rods. Gatenby and Ludford ('21) call this process dictyokinesis and the conditions they find in Helix are quite similar to those I find in Succinea. During the second maturation division the process is repeated in much the same manner as in the first (figs. 37 to 39), so that at the end of the second division there is in each spermatid approximately one-fourth the number of the Golgi rods found in the primary spermatocytes (fig. 43, etc.). Gatenby ('18) mentions the number of rods in the spermatids of different pulmonates. Among others, he finds six to twelve in Helix aspersa, two in Limax agresta, four to eight in Arion hortensis, six to eight in Helix nemoralis, five to seven in Helix rufescens, and six to fourteen in Testacella. Limax is the only form of which he is certain of an equal distribution of rods at the maturation divisions.

The extreme difficulty in demonstrating the Golgi elements during cell division has led Gatenby and Ludford ('21) to believe that some chemical change comes over the rods at this time. I have found that very long fixation in Flemming-

without-acetic is absolutely necessary for successful demonstrations of these stages. Some of my best preparations were fixed for three weeks. The foregoing authors think the chief reason for this difficulty in demonstrating these structures lies in the "withdrawal of some lipoid substance to which the heavier and more facile staining of the resting stage is due . . . . to serve some purpose during cell division."

The further fate of the Golgi apparatus is described under the section on the acrosome.

COMPONENTS OF THE SPERMATOZOON

The chief controversial points concerning the spermatogenesis of pulmonates have centered largely around the components of the spermatozoon. Many workers on pulmonates (Kleinart, '09; Buresch, '12; Gatenby, '17, '18) have not considered the final stages of the spermatozoon's development; others (Retzius, '06; Ballowitz, '90) have been more concerned with the final form of the sperm and have neglected the method of its origin and development; while only one worker (Bolles Lee, '04) has made an attempt to explain in detail the manner in which the sperm elements have arisen.

In this section I wish to point out the origin of the acrosome, the intranuclear rod, and the spiral structure of the spermatozoon as found in Succinea and to compare them with corresponding parts described for other forms.

A. Acrosome

The origin of the acrosome is intimately connected with the history of the idiozome and Golgi apparatus. In prosobranches a number of investigators, such as Meves ('03) on Paludina, Kuschakewitsch ('13, '21) on Vermetus, Schitz ('16) on Columbella, Ankel ('24) on Bythinia, and Hyman ('23) on Fasciolaria, have shown that the Golgi apparatus plus idiozome, or a part of it, develops directly into the definitive acrosome. In a series of papers Bowen ('20, '22, etc.) indicates that the acrosome in insects is formed in a

manner similar to that described for prosobranchs, and states that, with certain minor differences, this is probably the common plan of acrosomal formation throughout the animal kingdom.

In the case of pulmonates, however, there is no such unanimity of opinion regarding the acrosome. According to Platner ('85), the acrosome arises from a spermatid centrisome; Godlewski ('97 a and b) ascribes its origin to a nucleolus; while a later investigator, Prowezek ('01), believes that a special body, which he calls the 'Hauptkernkörperchen,' or 'Nebenkernkörperchen,' comes from the nucleus and takes part in the structure of the acrosome. Prenant ('89) states that the acrosome originates by certain transformations in the nuclear membrane itself—an observation which Kleinart ('09) confirms. The work of Bonnevie ('04 and '05) on Enteroxenos ostergreni agrees in many points with our present knowledge of the origin and development of the acrosome. She describes the Centrotheka (idiozome), or a part of it, as moving to the anterior pole from a region posterior to the nucleus, where it is found in early spermatids. Certain darkly staining granules which appear on this body are deposited on the nuclear membrane as the forerunner of the acrosome. She does not indicate the further fate of the Centrotheka remnant, nor does she point out any relationship between the Centrotheka and the Golgi apparatus. The description of Weigl ('12), working on Helix, in most particulars confirms those of Bonnevie. Gatenby ('18) thinks the acrosome comes from a special cytoplasmic body which has no relation to the idiozome and Golgi apparatus.

The formation of the acrosome in Succinea ovalis is quite similar to that described in certain prosobranchs by Kuschakewitsch ('13), Schitz ('16), and Ankel ('24). In early stages of the spermatid the idiozome and Golgi apparatus are found posterior to the nucleus, lying to one side or the other of the axial filament and the mass of mitochondria (figs. 47, 48, etc.). During later stages the complex moves slowly forward toward the anterior end of the spermatid

(figs. 50 to 53). While this migration is taking place, certain changes occur in the structure of the idiozome. At the end of the idiozome, usually at the place not covered with Golgi rods, appears a small vesicle enclosing a tiny granule (fig. 51, etc.). This granule, at first scarcely distinguishable, enlarges until it fills up most of the space of the vesicle (fig. 53). When the idiozome, which with the granule is now called the acroblast, reaches the anterior pole of the nucleus, the granule is deposited upon the nuclear membrane as the future acrosome (fig. 54). The Golgi remnant returns to the posterior region of the spermatid, where it can be seen in more mature

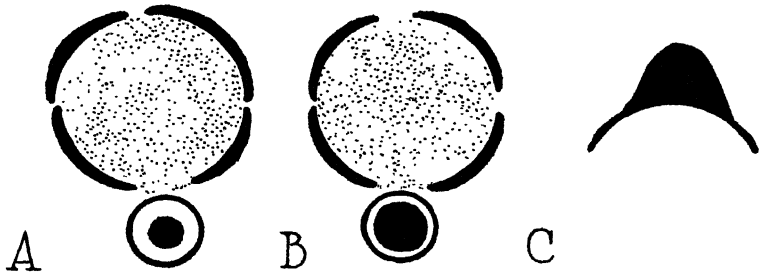

Fig. A Schematic representation of the acroblast of pulmonate spermatid, to show the origin of the acrosome. The idiozome is stippled, and the Golgi rods are shown in black. *A*, the acroblast at the time the vesicle and granule are first formed; *B*, a later stage, showing the enlargement of the granule to fill the space of the vesicle; *C*, the stage shortly after the granule has been deposited on the nuclear membrane. The granule assumes a dome shape and then the more pointed condition of the definitive acrosome.

spermatids (figs. 55 and 56, *g.re.*). The final fate of the Golgi remnant is found in the history of the spermatid remnant when the latter is cast off from the sperm tail (figs. 60, 61, and 73). In later stages the acrosome is seen as a small granule lying close against the anterior pole of the nucleus (figs. 55 and 56, *a*). It then becomes sharply pointed at one end (figs. 60 and 61). In its final differentiation the acrosome is very closely applied to the anterior end of the nucleus (fig. 67, etc.). In more mature stages the acrosome may be described as a lance-like, apical piece with its base closely applied to the wall of the nucleus (fig. 69). The formation of the acrosome is shown in schematic form in figure A.

B. Intranuclear rod

The structure of the intranuclear rod was first worked out in prosobranchs. It has been recognized for many years that in these forms the centriole is not confined to the middle piece and base of the sperm head, but that a prolongation of it extends through the head of the sperm. Chief among the investigators who have described the rod in prosobranchs have been Meves ('03), Stephan ('03), Kuschakewitsch ('13), Schitz ('16 and '20), and Hyman ('23). The manner or mode of formation of the rod, according to these observers, shows many variations among the different forms studied. However, in most of their accounts two elements are represented in the intranuclear rod, a centrosomal prolongation from the proximal centriole and an intranuclear canal which encloses the centrosomal rod.

Previous to the present account an intranuclear rod has never been described in pulmonates. In common with other pulmonates, Succinea ovalis has two centrioles in its spermatid stages (figs. 43 and 44). While these two centrioles lie close together in the early stages, they later separate, one, the proximal centriole, migrating toward the nucleus, and the other, the distal centriole, moving into that part of the spermatid which becomes the tail (figs. 50, 51, etc.). Between the two stretches the axial filament (figs. 45 and 46). The proximal centriole, on reaching the nuclear membrane, penetrates through the latter for a short distance (fig. 50). When the spermatid elongates a fine projection, the anterior axial filament grows forward from the proximal centriole through the spherical mass of oxychromatin which lies in the center of the nucleus (figs. 51 and 52). As soon as the filament has reached entirely through the nucleus, a portion of the oxychromatin extends along the filament to the anterior pole (fig. 53), and a little later another portion moves to the posterior pole of the nucleus (fig. 54). At first a bulging mass indicates the original position of the sphere of oxychromatin (figs. 54 and 55), but in time this bump is smoothed out and the whole forms around the rod a sheath of uniform

thickness (fig. 56 and others). Although this sheath gradually becomes very compact, the tiny filament can, in good preparations, be seen running through it (figs. 56 to 59). This entire structure, consisting of the anterior axial filament and its surrounding sheath of oxychromatin, is the intranuclear rod. When the nucleus of the spermatid finally contracts, the rod thickens and appears like those shown in figures 60, 61, and 62.

Some sort of an intranuclear rod has been reported for certain forms other than those already mentioned in this account. An intranuclear rod is described by Ballowitz ('90) in the coleopteran Morimus funereus; Thesing ('04), in Octopus defilippi, a cephalopod; Broman ('00), in Bombinator, a toad; Koster ('09), in the crustacean, Gammarus pulex, and Otte ('07), in Locusta.

In an examination of the literature the different cases can be classified into three groups:

1. In one group we may place those cases in which the centriole has been described as playing a very prominent part. This group includes most of the accounts to date (Meves, Stephan, Schitz, Hyman, Otte, Hickman). In all these investigations the chief element of the intranuclear rod in an outgrowth of the proximal centriole through the head of the sperm. Some (Koster, Otte) have described the rod as consisting entirely of this centrosomal outgrowth. Most of the others, however, mention an accessory structure, formed in diverse ways and called by different terms (intranuclear canal, intranuclear sheath, etc.) (fig. B, *A*).

2. The second class includes those who describe the nucleolus as forming the major, if not the entire portion, of the rod (Kuschakewitsch, Schitz) (fig. B, *B*).

3. The third class is made up of those workers who describe the formation of the rod from the idiozome (Thesing, Broman) (fig. B, *C*).

While it is impossible to harmonize all these accounts, most of the workers have regarded the centriole as playing the major part in the formation of the intranuclear rod. The

principal differences concern the intranuclear canal or sheath. Some of these differences are inexplicable, but many of them are no doubt due to the technique involved. The view of some (Meves, Stephan) that the intranuclear canal is formed from a vacuole in the longitudinal axis may not be so difficult to explain if one considers that the spermatid head at this time may stain in very different ways, depending upon the fixation, staining, etc. (Wilson, '25). Some stains show very

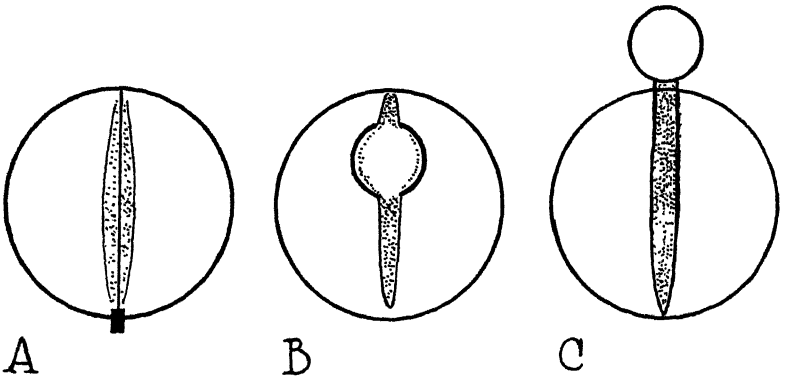

Fig. B Schematic representation of spermatid nuclei, to show the three methods proposed for the formation of the intranuclear rod. The rod is stippled. *A*, a type of formation (according to Meves, Stephan, Hyman, etc.) in which the rod is made up of two elements, a filament from the proximal centriole and a surrounding sheath; *B*, a second type (according to Kuschakewitsch and Schitz) in which the rod is derived from a nucleolus; *C*, a third type (according to Thesing and Broman), showing the origin from the idiozome. Note that in *B* and *C* the rod is supposed to consist of only one element which is probably analogous to the sheath described in *A*.

little of the nuclear center of the spermatid, while others may reveal definite structures in the same region. It is interesting to compare the accounts which describe the formation of the accessory structure of the rod from a nucleolus with the mode of origin in Succinea. In most essentials these descriptions agree, the chief difference being whether the mass in the nucleus is a nucleolus or something else. Schitz and Kuschakewitsch call the body a nucleolus, but I consider it simply a mass of oxychromatin. It may be that these

investigators confused the part played by the idiozome in the formation of the acrosome with the origin of the intranuclear rod. Thesing says that first the acrosome is formed from the idiozome, and then the rod originates from the acrosome. His account is radically different from that of others, and certainly there is no evidence in Succinea to justify such a view.

In those accounts which do not mention the centriole as taking part in the formation of the rod, the centrosomal element may not have been observed at all, and the rod described may really be the accessory structure. It is evident that the sheath is usually a more prominent structure than the rod, and the real difficulty is in demonstrating the outgrowth of the latter from the centriole. Thus Kuschakewitsch's description of the rod as being made up of the nucleolus alone fits in very well with other accounts of the accessory structure, leaving only the problem of whether a centrosomal rod is really present. In certain stages in the formation of the rod in Succinea it is much easier to see the intranuclear sheath than it is to demonstrate the anterior axial filament.

Although relatively few cases have been described so far, it is well to remember that the paucity of reports has probably been due to the scant attention given to this structure. When investigations are made for the express purpose of discovering the rod, it will no doubt be found in the spermatozoa of many other animals. It is possible that the intranuclear rod is as generally found in the sperm as, for instance, the centrioles.

This brings me to the real function and significance of the rod. Few investigators have ventured any definite conclusions along this line. According to Thesing, the rod is really a mechanical element of locomotion. He supports this view by instancing the undulating movement of the heads of living sperm. He also stresses the fact that the tube-like rod of the cephalopod spermatozoon has greater rigidity than a solid rod would have. Kuschakewitsch emphasizes the conclusion that the rod is an element of support. Hyman thinks that the structure may perform both these functions. Further-

more, he is of the opinion that the axial thread is the real active agent in the elongation of the head, and that the nucleus is passive while these changes are being brought about.

I also think that the rod represents in the nucleus what the axial filament does in the tail. Thus the rod, together with the axial filament, may be considered as the longitudinal axis around which the mature spermatozoon is built. This view seems to be borne out by the fact that both the anterior and posterior parts of the axial filament, together with the accessory structures, are early laid down in the spermatid and are present throughout spermiogenesis. The longitudinal axis may, in addition, perform the varying functions mentioned by previous observers.

C. Spiral structure

Although many workers have described the spiral form of the spermatozoon of pulmonates, few have made any attempt to explain the origin of the spirals. Bolles Lee ('04), in Helix pomatia, described the tail of the spermatozoon as made up of the axial filament lying embedded in a granular substance around which is closely wrapped a spiral ribbon. Retzius ('06, '10, etc.) was concerned chiefly with the mature spermatozoon of pulmonates and gave little attention to the mode of its formation. According to him, the tail of a pulmonate spermatozoon consists of two spiral threads compactly wound around each other. He describes one of these threads as being broad and clear and the other as being narrow and dark. He shows that in the immature stages the narrow, dark thread is the axial filament. As the spermatozoon approaches maturity the outer, larger thread loses its cylindrical nature and becomes ribbon-like. Retzius does not indicate the rôle of the mitochondria in the process.

My observations on Succinea ovalis are in accord with the work of the foregoing observers so far as the spiral nature of the spermatozoon is concerned. Spirals are easily demonstrated in both mature and late immature stages. The manner of the spiral formation, however, presents some difficulties.

The first indication of the spiral structure is found in the head region, where in stages like that shown in figures 66 to 68 a fairly pronounced twist is seen. This twisting continues until the head is thrown into a number of spirals (figs. 67 to 71). The spiral formation thus initiated in the head now continues down the tail. At this time the axial filament of the tail has been surrounded by a mitochondrial sheath, stages in the formation of which are shown in figures 53 to 67. Not all the mitochondria are thus found in the sheath, but a part, probably a fifth or sixth of the whole, is cast off in the cytoplasmic remnant (figs. 71 and 73). This axial filament with its mitochondrial sheath forms one of the two spiral elements of the tail. The other spiral element is composed of the less differentiated cytoplasm left by the sloughing off of the remnant (fig. 75), and is, in earlier stages at least, somewhat larger than the mitochondrial element. This second spiral element is wrapped around the darker, inner column, at first in loose spirals, but later more compactly (figs. 69, 72, 76, and 78). From my preparations I infer that the spiral elements remain together during the entire twisting process which begins only after the cytoplasmic remnant is found near the posterior tip of the sperm tail (fig. 68). Retzius ('06) does not think spirals exist in the head of pulmonate spermatozoa. Platner ('85 and '86) is of the opinion that the spiral structure of the head is confined to the immature stages of the spermatozoa. In the ducts where one should find mature spermatozoa I have found spirals in both head and tail (fig. 78).

In Succinea ovalis there is no uniformity of direction of the spirals. Retzius' ('06) statement that the spirals always go in a left-to-right direction from head to tail is not in conformity with the condition in Succinea. Out of many specimens examined, I find that the spirals may go in either a left or a right direction from head to tail, and that one condition is about as common as the other (figs. 68 and 69). Gatenby ('18) refers to the spirals in the spermatozoa of the pulmonate Testacella as arranged in "the opposite direction

to the hands of a clock and that there were no exceptions to this." He does not mention whether the head is spirally arranged or not, nor does he attempt to explain the origin of the spirals. Of course, it is possible that the spirals in the forms described by these investigators have such uniformity of direction and that Succinea ovalis is exceptional in this respect, or, in other words, this may be one of the numerous variations found in the spermatogenesis of even closely related forms.

Concerning the functional significance of the spiral arrangement of the sperm of pulmonates we are very much in the dark. Indeed, the great diversity of form in the spermatozoa of different groups in the animal kingdom cannot be explained at present in terms of differences in physiological function. The painstaking and careful work of Retzius and Ballowitz on a comparative study of different animal spermatozoa gives us very little insight into why they exhibit so many variations. It is difficult to find any significance in the direction of the spirals, owing to a lack of uniformity in this respect. Perhaps the real solution of the problem lies in a more careful study of the fertilization process than has as yet been made in pulmonates.

SPERMATID REMNANT

In early stages of the spermatids much cytoplasm is found surrounding the nucleus or future head of the spermatozoon (figs. 56 to 59). In later stages as the tail grows in length the mass of cytoplasm is found posterior to the head (fig. 60). By the time spirals are first observed in the head, the cytoplasmic remnant rests in the position shown in figure 68, where it remains during the subsequent lengthening and spiral twisting of the tail. This position marks the extreme posterior end of the tail proper, as the end piece, or naked axial filament, can be seen protruding from the mass (figs. 65 and 68). A broad zone of the remnant can be seen in regions where a considerable number of sperm are found in the same stages of development (fig. 71). As the sperm near the com-

pletion of their development these waste masses are completely sloughed off. The remnants in the alveoli are found lying free among the sperm or attached to them (fig. 73). In the sperm ducts the remnants are found along with the mature spermatozoa when the latter are stored in the ducts, ready to be discharged (fig. 75). Many of the fragments can always be found in the sperm ducts, and it is quite possible that a number of these are disengaged from the tails and simply lie free among them.

The cast-off bodies do not stain uniformly nor deeply with Flemming-hematoxylin, indicating disintegrative changes. They usually contain darkly staining particles of variable number and size. These may disintegrate to such an extent as to give a homogeneous appearance to the cytoplasm of the remnant (fig. 74). The shapes of the spermatid remnants are fairly constant, being oval and sac-like. It is impossible to determine just what they include. In most cases, however, they contain the Golgi remnant, the chromatoid body (in part of them), those mitochondria not used in the formation of the sheath of the axial filament, and probably other elements of obscure origin and significance. No doubt a considerable part of the whole remnant is made up of cytoplasm which has been left over in the process of the differentiation and final development of the sperm. The faintly staining and homogeneous character of the mass indicates this.

I have not been able to follow the fate of the remnants further. In most cases these fragments in the ducts are smaller than the original remnants—which shows that they are disintegrating. Hyman ('23), in the prosobranch Fasciolaria, thinks that the remnants are reabsorbed by the spermatozoa in situ, as he finds no evidence that the tail ball is cast off. On the other hand, it is possible that they may undergo the same fate as that described in the spermatogenesis of certain other forms, where the remnants are first sloughed off from the sperm tails and then are absorbed by the epithelial cells of the alveoli and ducts (Bowen, '22; Regaud, '01; Duesberg, '20), but I have no definite evidence of their fate in Succinea ovalis.

CHROMATOID BODY

The behavior of the chromatoid body in Succinea ovalis is quite similar to those cases described in many different forms by Wilson ('13), Payne ('16), Plough ('17), Carroll ('20), and Bowen ('22). In Succinea the structure is first seen in the primary spermatogonia, where it is very large and conspicuous (fig. 3). It is always single in the early stages of spermatogenesis, but later the body may be divided, indicating, perhaps, a fragmentation (fig. 29). But this division into two or more parts has no correlation with the maturation divisions, such as Ludford and Gatenby ('21) found in the mammals Mus and Cavia. These investigators describe the division of the body at each of the maturation divisions and thus each spermatid has a chromatoid body. In Succinea, after the spermatid begins to lengthen, this body is always found posterior to the head, and as the tail differentiates, it is discarded with the cytoplasmic remnant (fig. 75). Its fate in the remnants is perhaps like that of the other rejected structures, as it usually can be seen in various stages of disintegration. Its staining capacity is well maintained until it is finally broken up. No clue to the function of the chromatoid body could be found in this investigation, and, so far as I can determine, the body has never been described before by the numerous workers on pulmonates. That other pulmonates possess the body there cannot be the least doubt, for I have myself observed it in pulmonates other than Succinea.

MATURE SPERM

After the spiral formation of the head and tail has been completed, the entire spermatozoon presents a whorled appearance from acrosome to terminal filament (fig. 78). The tail is made up of an inner column, around which there is wrapped a prominent and rod-like spiral (fig. 72). The inner structure is slightly smaller than the outer and consists of the axial filament proper, darkly staining and rod-like, around which the mitochondrial sheath is wrapped in compact spirals. The nature of the sheath can be demonstrated only

by means of excellent technique. Bonnevie ('04) is of the opinion that the mitochondria really form a compact sheath such as I have found in Succinea ovalis. But she adds:

> Ob die Mitochondrien allein den Spiralfaden gebildet, oder ob sie es in Verbindung mit dem Zytoplasma getan haben, in welchem sie verteilt gefunden würden, darüber kann ich keine begründete Meinung aussprechen.

In Succinea the outer structure is the cytoplasmic body which has been transformed from a ribbon-like to a more cylindrical form in the process which ends with the sloughing off of the cytoplasmic remnant. Retzius ('06), in various pulmonates (Helix, etc.), also thinks that the outer spiral is composed of cytoplasm.

The heads of the mature spermatozoa stain with uniform intensity. They are sharply pointed, slightly sickle-shaped, and both acrosome and head proper blend so closely together that it is almost impossible to separate the two. Likewise, the tails take a much darker stain than in earlier stages, and the different elements composing the sperm can be made out only in those specimens which have been well destained. In order to be sure that the sperm are really mature, I have studied examples discharged from the alveoli into the sperm ducts.

Between the head and tail proper is a small middle piece about the same diameter as the tail. In its center and extending into the head are the darker proximal centriole and intranuclear rod. Compared with the sperm of most Mollusca, the middle piece in Succinea ovalis is quite small and inconspicuous (fig. 78). It is bounded at each end by a disk, the anterior one between the head and middle piece and the posterior one between the tail and middle piece. The latter is a convenient place for the head and attached middle piece to become disengaged from the tail, as in nearly all preparations many examples can be found where the heads have apparently been broken off accidentally from the tails (figs. 76 and 77).

The tail tapers slightly from the middle piece to the region of the tail filament. The latter structure is the naked axial

filament which in early stages of spermiogenesis extends beyond the region of the cytoplasmic body (see earlier figures). However, it is difficult to distinguish any point of demarcation between the tail proper and the tail filament in mature sperm, for the one blends into the other quite gradually.

Mature sperm of Succinea are unusually long, averaging about 420 μ in length. Of this length the head and middle piece make up 25 μ, while the greatest diameter of the head is about 5 μ.

CONCLUSIONS

1. A study is made of the maturation stages of Succinea ovalis Say.

2. The chromosomes, the diploid number of which is forty, are small rod-like bodies of varying size.

3. Of the cytoplasmic inclusions, the mitochondria and the Golgi apparatus are traced in detail, and their part in the formation of the sperm is shown.

4. Mitochondria first appear in the male progerminative cells as small granules. In the succeeding divisional stages they are approximately equally distributed among the daughter cells.

5. During the maturation stages the granules are arranged in short threads.

6. Some of the mitochondria form a sheath around the axial filament; the remainder are sloughed off with the spermatid remnant.

7. A Golgi apparatus can be identified in the secondary spermatogonia, where the rods are more or less scattered. From fifteen to twenty rods can be counted in the spermatocytes. These are approximately equally divided between the daughter cells at the two maturation divisions, so that each spermatid has about one-fourth of the original number of rods found in the spermatocytes.

8. At dictyokinesis there is no fragmentation of Golgi rods, but they are distributed intact.

9. The mode of formation of the apical body is similar to that recently described for many other forms.

10. An intranuclear rod, consisting of an anterior axial filament enclosed by a sheath of oxychromatin, is formed in the early stages of the spermatid. In the mature sperm the rod is seen as a very compact structure extending entirely through the head.

11. The functions of the rod as the main longitudinal axis of the sperm are discussed.

12. Mature sperm possess a spiral arrangement both in the head and tail regions. The inner column of the tail is the axial filament plus its surrounding sheath of mitochondria; the outer spiral is composed of cytoplasm.

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EXPLANATION OF PLATES

All the figures were drawn at table level with a camera lucida. Many fine details were put in free-hand. Unless otherwise noted under the appropriate explanation, the drawings were made with the use of a 2-mm. Leitz apochromatic objective and a no. 12 ocular. The figures have been reduced one-fifth in reproducing them here. The method of fixation and staining employed in the original preparation is indicated in each case.

ABBREVIATIONS

a., acrosome
a.af., anterior axial filament
a.c., acroblast
a.f., axial filament
a.l., Ancel's layer
c., centriole
ch.b., chromatoid body
c.r., cytoplasmic remnant
g.a., Golgi apparatus
g.c., germinal epithelial cell
g.r., Golgi rod
g.re., Golgi remnant
id., idiozome
i.r., intranuclear rod
m., mitochondria
m.b., midbody
m.p., middle piece
m.p.c., male progerminative cell
n.c., nurse cell
p.sg., primary spermatogonium
s.r., spindle remnant
s.sg., secondary spermatogonium

PLATE 1

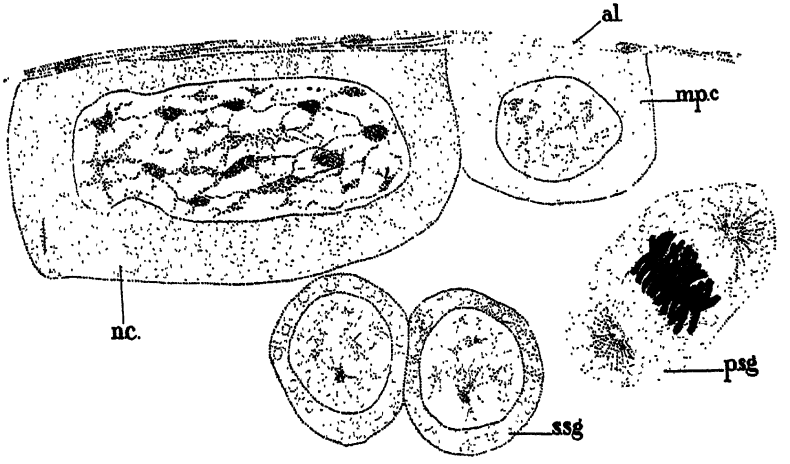
EXPLANATION OF FIGURES

1 and 2 Sections through small portions of an acinus, showing some germ elements free in the lumen and others still in the germinal epithelium. (Flemming-without-acetic and iron hematoxylin.)

3 Primary spermatogonium. First appearance of chromatoid body. (Flemming-without-acetic and iron hematoxylin.)

4 Section of the germinal epithelium during a period when the gland is very inactive. (Flemming-without-acetic and iron hematoxylin.)

5 Prophase stage in the first spermatogonial division. Note the long, curved chromosomes. (Bouin and iron hematoxylin.)



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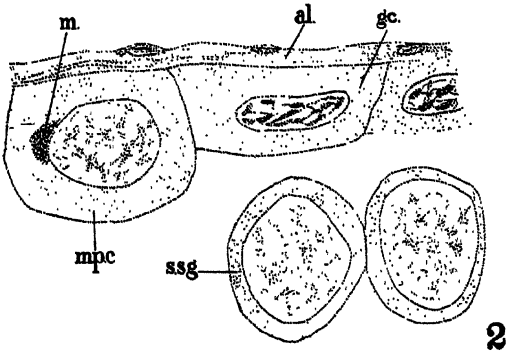

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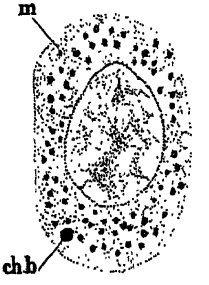

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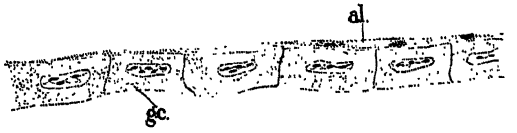

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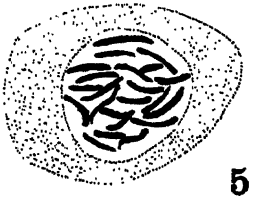

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PLATE 2

EXPLANATION OF FIGURES

6 Metaphase plate of first spermatogonial divisions. (Bouin and iron hematoxylin.)

7 Anaphase of spermatogonial divisions. Mitochondria do not clump together in rows as they do in the maturation divisions. (Flemming-without-acetic and iron hematoxylin.)

8 and 9 Resting secondary spermatogonia. Golgi rods are seen scattered at one side of the nuclei. (Flemming-without-acetic and iron hematoxylin.)

10 and 11 Resting primary spermatocytes. The idiozome in figure 10 is shown without the Golgi rods, as the latter have been destroyed by the fixative. (10, Flemming and iron hematoxylin; 11, osmic acid and iron hematoxylin.)

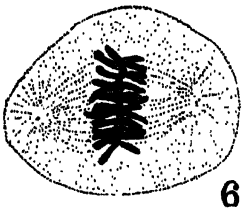
12 Leptotene stage with cytoplasmic elements, except idiozome, removed. (Flemming and iron hematoxylin.)

13 and 14 Presynaptic bouquet with cytoplasmic elements preserved. (13, Flemming and iron hematoxylin; 14, Flemming-without-acetic and iron hematoxylin.)

15 Bouquet stage, showing the process of synapsis. Some of the threads are paired. (Flemming and iron hematoxylin.)

16 Postsynaptic bouquet. All the threads are paired. (Flemming and iron hematoxylin.)

17 Resolution phase of pachytene threads. (Flemming and iron hematoxylin.)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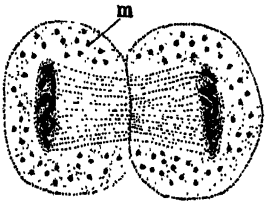

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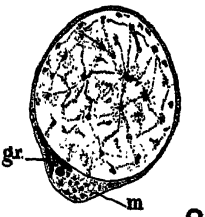

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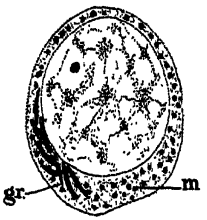

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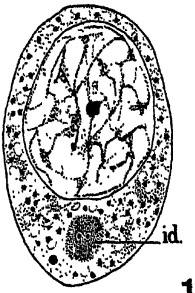

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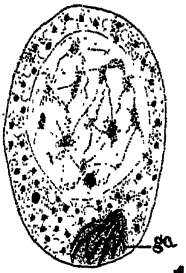

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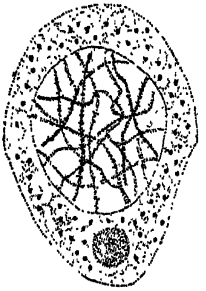
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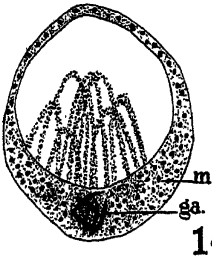

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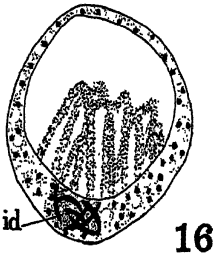

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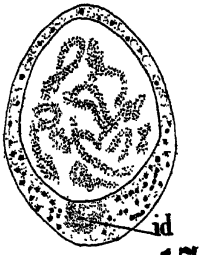

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PLATE 3

EXPLANATION OF FIGURES

18 A slightly more advanced stage than figure 17, showing further resolution. (Flemming-without-acetic and iron hematoxylin.)

19 to 23 Stages in the formation of the tetrads. Mitochondria are beginning to appear in the form of rodlets, or chains. The Golgi rods have separated into two masses in figure 23. (Flemming-without-acetic and iron hematoxylin.)

24 and 25 Side view of metaphase plate of first maturation division. Golgi apparatus not preserved. (Flemming and iron hematoxylin.)

26 and 28 Polar views of metaphase plate. Cytoplasmic elements not preserved by fixation in figure 28. (26, Flemming-without-acetic and iron hematoxylin; 28, Bouin and iron hematoxylin.)

27 Side view of metaphase plate. Golgi rods are seen at each of the poles. (Osmic acid.)

29 Anaphase of first maturation division. (Bouin and iron hematoxylin.)

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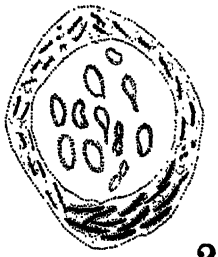

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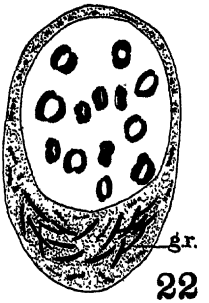

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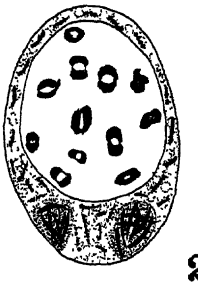

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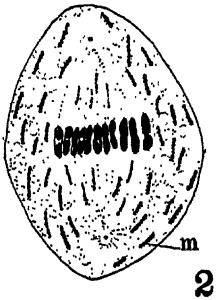

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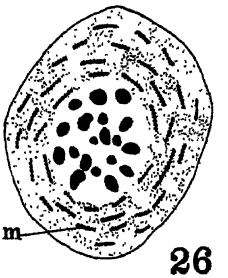

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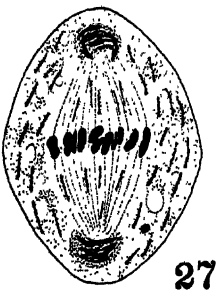

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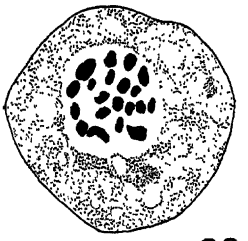

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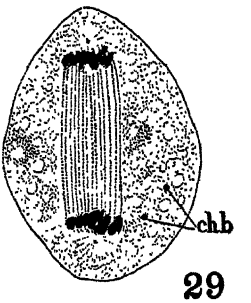

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PLATE 4

EXPLANATION OF FIGURES

30 Anaphase of first maturation division. Mitochondria gathered into short chains. Chromatoid body is seen near one chromosome plate. (Flemming-without-acetic and iron hematoxylin.)

31 Anaphase of first maturation division, showing very compact chromosomal plates. (Flemming and iron hematoxylin.)

32 A slightly later stage than figure 31. Chromosomal plates loosening up. (Flemming and iron hematoxylin.)

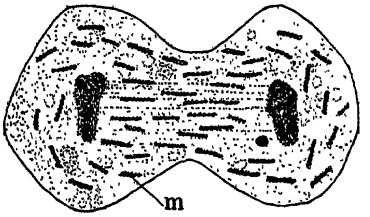
33 Secondary spermatocytes still connected by spindle remnant. Nuclear membrane is reconstructed. (Flemming and iron hematoxylin.)

34 and 35 Secondary spermatocytes, showing cytoplasmic inclusions. (Flemming-without-acetic and iron hematoxylin.)

36 and 37 Side view of metaphase plate, second maturation division. In figure 37 Golgi rods are seen at each of the poles. (36, Flemming strong; 37, Flemming-without-acetic.)

38 Polar view of metaphase plate, second maturation division. (Flemming and iron hematoxylin.)

39 Anaphase of second maturation division. Golgi rods are found close to the chromosomal plates. (Flemming-without-acetic and iron hematoxylin.)



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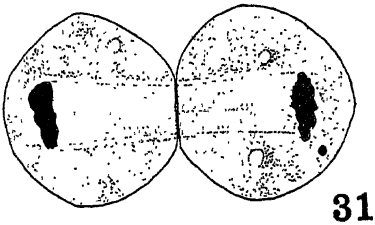

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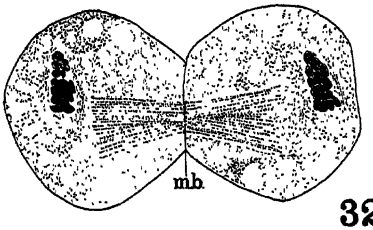

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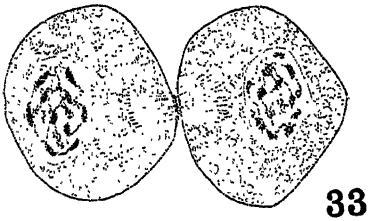

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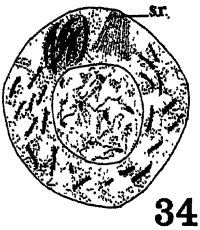

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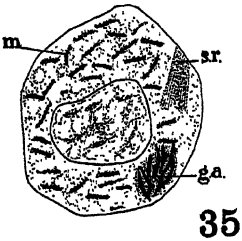

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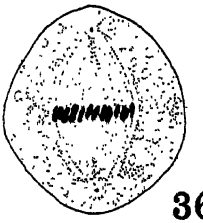

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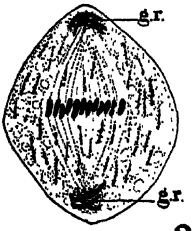

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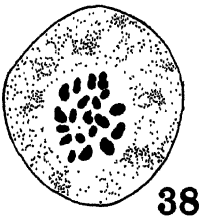

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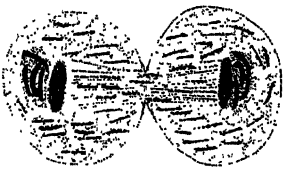

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PLATE 5

EXPLANATION OF FIGURES

40 A slightly later anaphase stage. Chromatoid body is in one of the daughter cells. Fixative did not preserve other cytoplasmic structures. (Flemming and iron hematoxylin.)

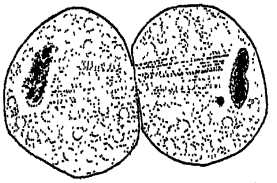
41 and 42 Young spermatids still connected by spindle bridge. Nuclear membranes have been formed and the chromosomal plates have loosened up. Figure 42 was drawn with a 4-mm. Bausch & Lomb apochromatic objective and no. 10 ocular. (Bouin and iron hematoxylin.)

43 and 44 Young spermatids with cytoplasmic inclusions. The centrioles are seen close together. (Flemming-without-acetic and iron hematoxylin.)

45 Young spermatid, showing beginning of axial filament. Golgi apparatus and mitochondria not preserved. (Bouin and iron hematoxylin.)

46 to 49 Young spermatids, showing early changes in nuclear substance. (Flemming-without-acetic and iron hematoxylin.)

50 to 54 Series of spermatids, showing the formation of the intranuclear rod and the acrosome. (Flemming-without-acetic and iron hematoxylin.)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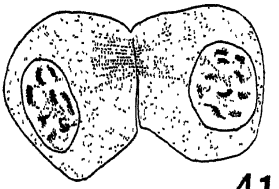
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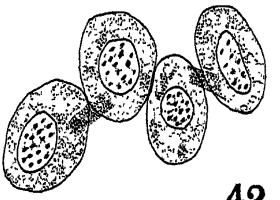
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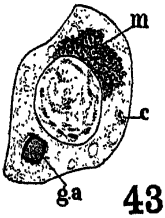

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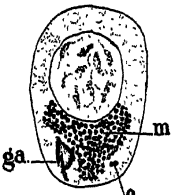

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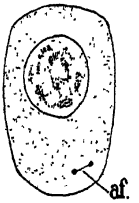

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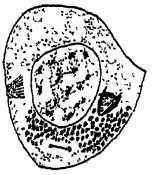
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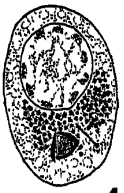
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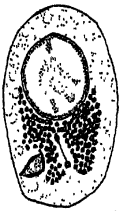
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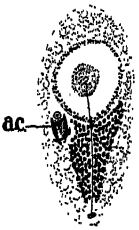

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PLATE 6

EXPLANATION OF FIGURES

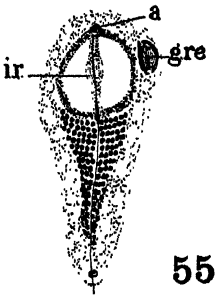
55 and 56 Young spermatids, showing intranuclear rod and acrosome laid down. (Flemming-without-acetic and iron hematoxylin.)

57 to 59 Spermatid nuclei, showing the breaking up and condensation of the chromatin. The nuclear sap is extruded during this process. (Flemming-without-acetic and iron hematoxylin.)

60 and 61 Slightly older spermatids, with chromatin highly condensed. The cytoplasm or spermatid remnant is seen as a bulging mass at posterior side of the nucleus in figure 60. In figure 61 the mass is found posteriorly, carrying with it the Golgi remnant. (Flemming-without-acetic and iron hematoxylin.)

62 Older spermatid with head more elongated. The intranuclear rod is faintly seen. (Flemming-without-acetic and iron hematoxylin.)

63 and 64 Heads of older spermatids, showing in figure 64 the beginning of a spiral in the head. Intranuclear rods quite distinct and middle pieces fully formed. (Flemming-without-acetic and iron hematoxylin.)



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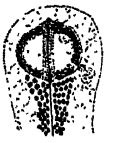

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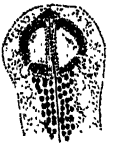

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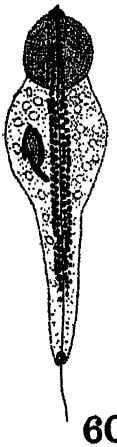

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PLATE 7

EXPLANATION OF FIGURES

65 A spermatid of about the same stage as that of figure 63, but showing a spiral going in the opposite direction. (Flemming-without-acetic and iron hematoxylin.)

66 and 67 Heads and part of the tails of still older spermatids, showing spiral formation. (Flemming-without-acetic and iron hematoxylin.)

68 and 69 Spermatids with still more advanced stages of spiral formation. (Flemming-without-acetic and iron hematoxylin.)

70 A nurse cell with attached spermatid heads. About the same side as those in figure 71. (Flemming-without-acetic and iron hematoxylin.)

71 Group of very mature spermatids with spiral formation almost completed. Characteristic arrangement of cytoplasmic remnants at this stage. Both figures 70 and 71 were drawn with 4-mm. Bausch & Lomb apochromatic objective and no. 10 ocular. (Flemming-without-acetic and iron hematoxylin.)

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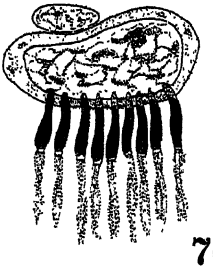

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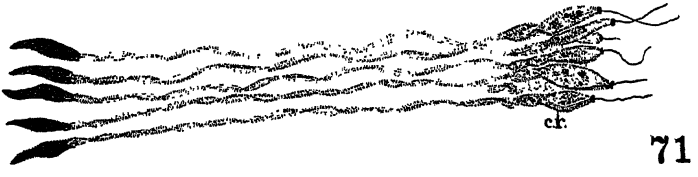

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PLATE 8

EXPLANATION OF FIGURES

72 Older spermatid, showing nature of spirals. Full length of tail not shown. (Flemming-without-acetic and iron hematoxylin.)

73 A bunch of cytoplasmic remnants, most of which are still attached to the sperm tails. (Flemming-without-acetic and iron hematoxylin.)

74 A cytoplasmic remnant just about to be sloughed off from the sperm tail. (Flemming-without-acetic and iron hematoxylin.)

75 Cytoplasmic remnants sloughed off from the sperm tail. (Flemming-without-acetic and iron hematoxylin.)

76 Sperm heads and attached middle pieces broken off from tails. (Flemming-without-acetic and iron hematoxylin.)

77 Portion of sperm tail, showing region where middle piece is commonly disjointed from the tail. (Flemming-without-acetic and iron hematoxylin.)

78 Mature spermatozoon from the sperm duct. Head has been very much destained. (Osmic acid and iron hematoxylin.)

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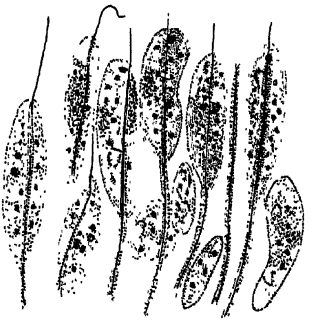

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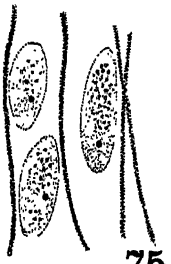

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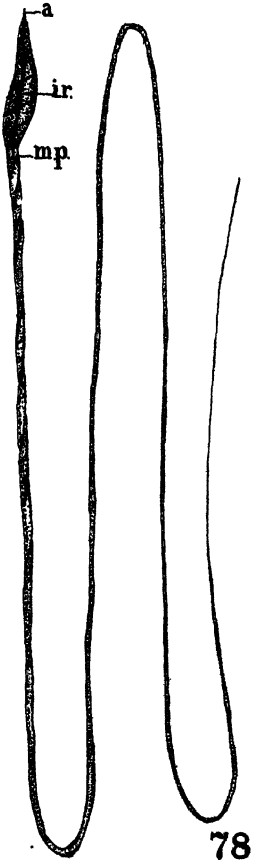

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THE MORPHOLOGY OF SPIRONYMPHA, WITH A DESCRIPTION OF A NEW SPECIES FROM RETICULITERMES HESPERUS BANKS

GEORGE V. E. BROWN

Zoölogical Laboratory, University of California

TWO HELIOTYPE PLATES (NINE FIGURES)

AUTHOR'S ABSTRACT

Spironympha is discussed as a valid genus. It was described by Koidzumi ('16); later it was redescribed by the same author as Microspironympha ('21). Therefore, according to the rules of nomenclature, it is Spironympha.

The genus is compared with the related genera: Spirotrichonympha, Holomastigotes, and Microjoenia.

Spironympha is characterized by four flagellar bands which are spirally wound around the anterior part of the body; these bands occur only in the anterior end, whereas in Spirotrichonympha they extend almost to the posterior end. The parabasals are few in number, and they are attached to the basal granules of the flagellar bands; the anterior end is clear and almost free from cytoplasmic granules; and there are twenty to thirty anterior flagella which are attached to the base of the centroblepharoplast or to the basal granules of the flagellar bands. An axostyle is present.

No centrosome occurs within the nucleus, but the centroblepharoplast has this kinetic function.

Spironympha ovalis is described as a new species. It is ovoid; the average size is 38 μ to 44 μ. An axostyle is present. The host is Reticulitermes hesperus Banks.

CONTENTS

Introduction 291
Material and technique 292
General morphology 293
Spironympha ovalis sp.nov. 295
Discussion 296
Summary 299
Literature cited 299

INTRODUCTION

Spironympha was first described and figured by Koidzumi ('17) in Japanese. Later ('21), he referred to this genus as Microspironympha. MacKinnon ('27) has described a species from Leucotermes tenuis as Microspironympha elegans. In all probability, this species should be placed in the genus Spirotrichonympha Grassi, because of the structure and the arrangement of its flagellar bands. Bernstein ('28) reported

the occurrence of Microspironympha porteri Koidzumi in Hodotermes miergabicus from Turkestan.

Duboscq and Grassé ('28) believe that Spironympha (= Microspironympha) and Microjoenia should be considered as the young forms of Spirotrichonympha. After a careful study of Spironympha, Spirotrichonympha, and Microjoenia, the writer has decided that these should stand as valid genera. There are not enough intergrading forms to justify the contention of Duboscq and Grassé. These three genera show marked differences in size, but they always possess generic characteristics of a definite nature.

This work was begun at the University of California and it was completed at the Johns Hopkins University. The writer wishes to take this occasion to express many thanks to the faculties of both institutions for their criticisms and general cooperation.

MATERIAL AND TECHNIQUE

The termites used in this investigation was Reticulitermes hesperus Banks. These were obtained from Strawberry Cañon on the University of California campus. This species of termite is quite common around the San Francisco Bay region of California.

After the termites were collected, they were placed on a filter-paper diet for about a week and many small flagellates were noticed to be abundant in the intestinal contents. Among these Spironympha occurred in abundance.

For fixation, the slides were covered with a very thin coating of albuminate fixative and then the intestinal contents were teased out into a small drop of normal saline solution. Various fixing reagents were tried, but the best were found to be Champy's, strong Flemming's without acetic, and Yabroff's modification of Da Fano (Yabroff, '28); Heidenhain's iron-alum haematoxylin was used as a nuclear stain. Light green proved to be the best counterstain.

GENERAL MORPHOLOGY

Two forms of Spironympha are present in the lumen of the intestine of Reticulitermes hesperus. These have been determined to be Spironympha porteri Koidzumi and a new species which will be described later.

Spironympha differs from the related genus Spirotrichonympha in structure, shape, and behavior. It swims more like Holomastigotes; that is, with a slow 'shuffle.' Spirotrichonympha swims rapidly and is constantly turning its anterior end about. The anterior portion of Spironympha is usually blunt when compared with the long, sharp, anterior end of Spirotrichonympha. Likewise, the form of Spironympha is more top-like than any of its close relatives.

Size is a variable factor in both genera. Both genera have four flagellar bands, but Spirotrichonympha has a granular layer between the bands, whereas Spironympha has an anterior end which is almost free from granules.

Koidzumi ('21) describes Spirotrichonympha leidyi which measures 15 to 50 μ × 18 to 30 μ, whereas he states that Microspironympha porteri measures 20 to 55 μ × 10 to 30 μ. This in general agrees with my observations; each species is variable in size; this is caused by autotomy or the sloughing off of a part of its body. Autotomy occurs frequently in both genera and the entire posterior end may be thrown off. This adaptation causes a marked change in size, but regardless of this, the generic characteristics are retained. Forms seem to occur in both genera in which the axostyle is present and, also, other types are known where it may fail to occur.

Spironympha is easily determined in the living state, because there are four flagellar bands which are wound around the body and these are not as heavy as the flagellar bands of Spirotrichonympha and they are often deep in the endoplasm. Also, the flagellar bands of Spironympha are short and occur only in the anterior portion. The parabasals of Spironympha are few in number; they are small and spherical and become more numerous toward the end of the flagellar bands, whereas those of Spirotrichonympha are large,

angular, and numerous. The basal granules of Spironympha are often large and numerous; these occur along the flagellar bands, whereas the basal granules of Spirotrichonympha are small. The endoplasm in the anterior end is usually quite clear when compared with that of Spirotrichonympha (Koidzumi, '21). The nucleus is ovoid in Spironympha and it contains a large endosome which often becomes fragmented into two or more parts (Koidzumi, '21; Bernstein, '28).

Bacteria are often attached to the posterior end of the body. These are usually stout and when separated from the body they swim off. Bacteria of this type have been repeatedly confused with the flagella by various workers.

The form of Spironympha porteri which occurs in Reticulitermes hesperus is top-shaped or spindle-shaped. It measures 35 to 42 μ in length and 18 to 22 μ in width. It has a heavy axostyle which projects as a point at the posterior end of the flagellate (fig. 4). There are four flagellar bands present and each of these makes one or two turns around the body. When the animal is viewed from a surface focus, these bands appear as a continuous spiral (as figured by Koidzumi, '21). The parabasals are small and ovoid in this species and they are not numerous (fig. 2). The parabasal bodies are attached to the basal granules on the flagellar bands by very fine strands. The basal granules are large and numerous. The flagella originate from the centroblepharoplast and also from the basal granules on the flagellar bands.

The axostyle often contains a dark chromatic strand which lies near its center. The centroblepharoplast has a small 'operculum' (Kofoid and Swezy, '15) which is similar to that of the trichonymphids (fig. 2).

A rhizoplast connects the centroblepharoplast with the nucleus. A large homogeneous endosome occurs in most of the flagellates studied. It is sometimes fragmented, as described by Koidzumi ('21).

The anterior end of Spironympha porteri is hyaline in both stained and living material (fig. 4).

Bernstein ('28) has described Spironympha porteri to occur in Hodotermes miergabicus of Turkestan. Her figures show a marked difference in form, size, and shape, and, in fact, they seem to be different animals entirely from the Protozoa described in this paper. The flagellate she has described seems to possess many features which are different from the flagellates found in Reticulitermes, and the writer thinks it safe to say that it does not conform with the form of Spironympha porteri found in Reticulitermes hesperus.

Different genera of termites have been studied and it has been noticed that the fauna of these different termites differ greatly; in fact, each termite genus seems to be inhabited by forms of Protozoa which are not found in the other termites. This alone, as well as the structural differences involved, causes me to believe that the forms studied by Bernstein ('28) are not Spironympha porteri Koidzumi, but that it belongs possibly to some related species.

Spironympha ovalis sp.nov. (figs. 1 and 5, 6 to 9)

This species is larger than Spironympha porteri Koidzumi. In length it measures from 36 to 48 μ; in width, 32 to 52 μ. The average length is about 44 μ; the average width, 40 μ. The body shape is ovoid. An axostyle may or may not be present. In either case the contour of the body remains ovoid.

Four flagellar bands are present and these are tightly wound around the anterior end. These bands are attached to the centroblepharoplast, which is tubular. An 'operculum' is present as a round knob on the anterior end. The centroblepharoplast is capable of being drawn into the body (fig. 9).

Parabasal bodies are large, but few in number, and occur near the ends of the flagellar bands. The parabasals are attached by short strands to the basal granules (fig. 1). The anterior flagella are attached to the basal granules of the flagellar bands or to the base of the centroblepharoplast.

From an apical view these four flagellar bands appear as a spiral. The bands are shorter than those of Spironympha porteri and they occur only in the anterior end (fig. 1).

The nucleus is usually spherical, with a large ovoid endosome of a homogeneous nature. The nuclear membrane often has chromatin encrusted around it. The axostyle is rarely attached to the posterior end of the body as it is in Spironympha porteri, but it is of a more or less fibrous nature. Anterior to the nucleus it is attached to the tubular portion of the centroblepharoplast.

DISCUSSION

The genus Spironympha has recently been discussed as to its validity by Duboscq and Grassé ('28). They believe this genus to be a stage in the life cycle of Spirotrichonympha and they have described and figured types which they believe to be intergrading forms between Microjoenia, Microspironympha (= Spironympha), and Spirotrichonympha. The writer finds that these so-called intergrading types belong to a new genus. Although they are closely related to these genera, they usually occur in termite hosts where some of the other genera are not found. Therefore, it seems doubtful that they are the young forms of Spirotrichonympha.

Koidzumi ('21) believed that Spironympha was closely related to the trichonymphids and placed it in the subfamily Holomastigotinae Grassi. It seems to be a form between Holomastigotes and Spirotrichonympha.

Reichenow ('28) believes that Spironympha is a separate genus from Spirotrichonympha. He places this genus under the subfamily Holomastigotinae of Grassi.

Axostyle

The axostyle of Spironympha was figured by Koidzumi ('16–'17), but he failed to include it in his description. MacKinnon ('27) described an axostyle in Microspironympha elegans. Bernstein ('28) describes an axostyle; and, recently, Duboscq and Grassé ('28) have shown that an axostyle is usually present in Spirotrichonympha; and they describe an axostyle to occur in the 'microspironymphid' forms of Spirotrichonympha.

Spironympha porteri, found in Reticulitermes, shows a definite axostyle which may be absent for a short time during the early prophase. In nearly all of the material killed with an osmic-acid fixative, it was present. In material impregnated with osmic acid there is a band around the posterior end of the axostyle (fig. 8).

Centroblepharoplast

The centroblepharoplast of Spironympha is more or less tubular, according to Koidzumi ('17, '21). Attached to the centroblepharoplast are the rest of the organelles of the neuromotor system. This neuromotor complex is composed of numerous anterior flagella, four flagellar bands (with parabasals and basal granules), the axostyle, and the rhizoplast of the nucleus.

The flagellar bands

The flagellar bands are four in number, as described by Reichenow ('28). These bands are of a definite number, and are not from six to eight as described by Koidzumi ('20). Usually the flagellar bands make two turns each around the body, and from an external or surface view these give an impression that a continuous spiral is present (fig. 6). A change in focus will show that this spiral is due to the flagellar bands winding around the body.

The endoplasm is clear and almost free from cytoplasmic granules in the region of these bands (Koidzumi, '21), whereas Spirotrichonympha has a granular layer between the flagellar bands (Grassi, '17).

Koidzumi ('17, '21) described the occurrence of the rows of basal granules. He believed these rows of granules to be of a variable number. The writer finds that the basal granules of Spironympha ovalis are attached to the four flagellar bands. The parabasals were described by Duboscq and Grassé ('28) to occur in Spirotrichonympha. I find that the parabasal bodies of Spironympha are similar to those described by Duboscq and Grassé, except that they are

smaller, more ovoid, and fewer in number than those in the related genus Spirotrichonympha.

The flagella

The flagella are almost as numerous as those of Spirotrichonympha. However, most of the flagella are anterior and they are not divided into zones of short and long groups as is characteristic of many of the trichonymphids (Kofoid and Swezy, '29).

Nucleus

The nucleus is ovoid, with a large endosome which may often be fragmented into two or more pieces (Koidzumi, '21). Bernstein ('28) describes a centrosome to occur within the nucleus, but it is possible that this is only a piece of chromatin. In fact, the centroblepharoplast has the function of a centrosome (Kofoid and Swezy, '19).

Endoplasm

The endoplasm in the anterior end of Spironympha is not as granular as that of Spirotrichonympha or Holomastigotes. The anterior end is quite clear, whereas the posterior end is often full of granules. These are never as large, nor are they as numerous, as the granules in the endoplasm of Holomastigotes.

Spironympha ovalis sp.nov.

Diagnosis. Xylophagous hypermastigotes of a ovoid shape which are 44 μ in length and 39 to 40 μ in width. Axostyle is usually present. Four flagellar bands occur in the anterior end. These are close together and attached to the centroblepharoplast, and they are wound around the anterior end in a definite manner. The centroblepharoplast is tubular. The parabasal bodies are large, ovoid, and few in number. The parabasals are suspended from the basal granules of the flagellar bands. Twenty to thirty anterior flagella are

attached to the basal granules or the centroblepharoplast. Bacteria may be attached to the posterior part of the body. The nucleus is ovoid; it is situated anteriad and rarely is the large spherical endosome fragmented. The host is Reticulitermes hesperus Banks.

SUMMARY

1. Spironympha ovalis is described as a new species occurring in Reticulitermes hesperus Banks.

2. Spironympha is described and discussed as a valid genus. Distinctive characteristics of the genus are pointed out.

3. Spironympha is compared with the related genera: Spirotrichonympha, Holomastigotes, and Microjoenia.

4. Spironympha ovalis is ovoid and rounded posteriorly. The average size is 44 μ × 38 μ.

5. Four flagellar bands are closely wound around the body in the anterior region. The parabasals are ovoid and few in number and they are suspended from the basal granules of these flagellar bands.

6. The centroblepharoplast is tubular and no 'operculum' is present.

7. The nucleus is ovoid and situated anteriorly. It is usually attached to the centroblepharoplast by a rhizoplast.

8. The axostyle is usually present. It originates from the centroblepharoplast and continues past the nucleus to the posterior part of the body. In many cases it ends freely in the endoplasm.

9. Twenty to thirty anterior flagella occur. These are attached to the centroblepharoplast or to the basal granules of the four flagellar bands.

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PLATES

All figures are camera-lucida drawings of Spironympha porteri Koidzumi and Spironympha ovalis sp.nov. from Reticulitermes hesperus Banks. Figures 1, 2, 3, 4, 7, and 9 were fixed in Champy's fluid. Figures 5 and 6 were fixed in Flemming's fluid (without acetic). All figures mentioned above are drawn from material stained in Heidenhain's iron-haematoxylin. Figure 8 was stained with Yabroff's modification of Da Fano.

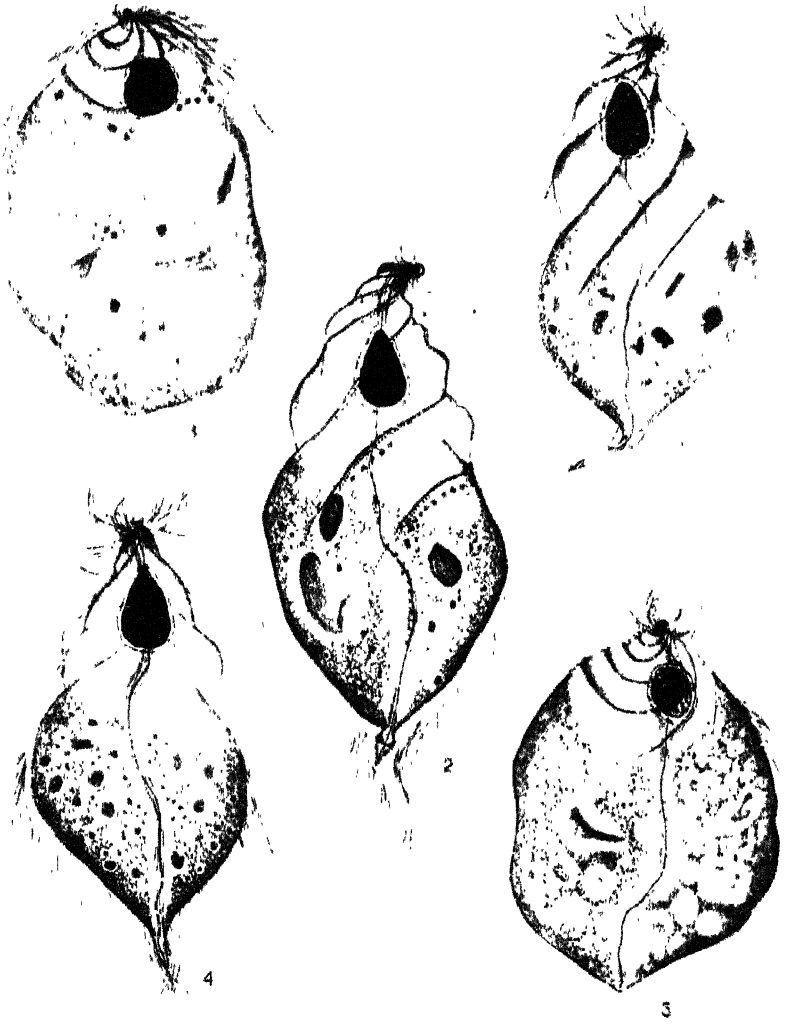
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PLATE 1

EXPLANATION OF FIGURES

Figures 1 and 5 are Spironympha ovalis sp.nov. Figures 2, 3, and 4 are Spironympha porteri Koidzumi.

1 Surface view showing parabasal bodies attached to the basal granules on the flagellar bands.

2 Drawn as a transparent object to show the four flagellar bands. Note axostyle has endostylic granules and an axostylic band occurs around the posterior end of the axostyle.

3 Surface view shows two of the flagellar bands. Note the anterior is free from granules.

4 Drawn from a focus near the center of the body. The axostyle is fibrous.

5 A surface view of Spironympha ovalis. Note axostyle and two of the flagellar bands are shown.

PLATE 2

EXPLANATION OF FIGURES

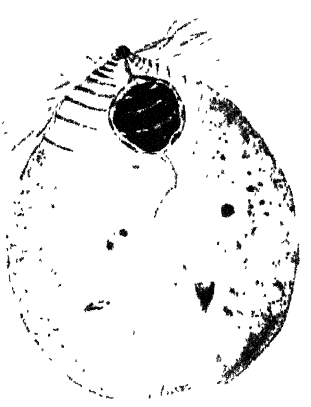
Figures 6, 7, and 9 are Spironympha ovalis sp.nov. Figure 8 is Spironympha porteri Koidzumi.

6 Shows the spiral appearance of the flagellar bands. The axostyle is present.

7 Parabasals are shown suspended from the basal granules.

8 The large disc-shaped bodies are dictyosomes. The small spherical bodies are mitochondria.

9 Centroblepharoplast is withdrawn into body. Note rhizoplasts connecting the nucleus with the centroblepharoplast.



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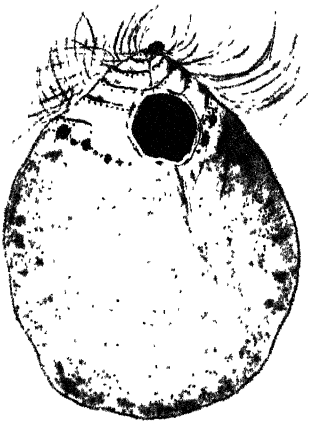

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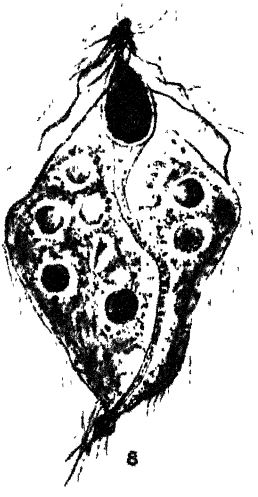

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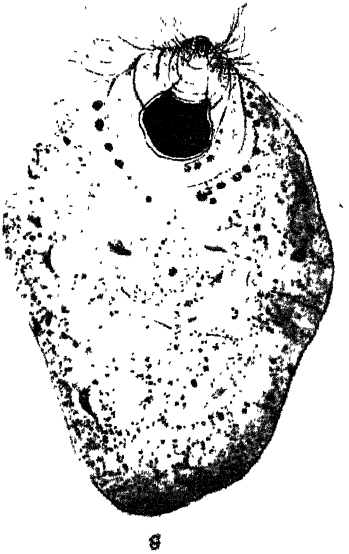

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V.E. Brown del.

OBSERVATIONS ON THE 'RENAL-PORTAL' PERFUSION IN ETHERIZED BIRDS

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ONE FIGURE

AUTHOR'S ABSTRACT

This investigation was made to demonstrate the direction of the current of a perfusing fluid (hence that of blood in nature) inside the so-called 'renal-portal' vein of birds and to determine if this vein has any fine capillaries in the kidney substance. A domesticated male duck was anaesthetized with ether, and a warm saline (mixed with a little urea and urine) was passed through the aorta. The 'renal-portal' vein was also perfused with the same fluid through the left internal iliac vein. At first the kidneys actively secreted semi-solid urine, but gradually the strength of the latter varied from a milky to a watery fluid.

Later, a warm carmine solution was perfused through the left internal iliac vein, and the path of the dye could be easily traced along the whole length of the left renal afferent (left 'renal-portal' vein) and its final exit through the postcaval vein. The posterior lobe of the left kidney was partially tinged with red, probably due to diffusion, since the kidney substance should have taken a uniform red hue if there was any definite capillary system. The coccygeomesenteric vein contained no dye.

These results (coupled with actual caliber measurements of the two 'renal-portal' veins in duck and pigeon examined, the calibers of these veins increasing gradually postero-anteriorly) indicate that: 1) blood flows anteriorly in the 'renal-portal' vein; 2) this vein does not break up into capillaries in the kidney substance, but receives larger affluent veins; 3) there is no 'renal-portal' system in birds; 4) the urine secreted by birds is always semisolid.

CONTENTS

Introductory 309
Modus operandi and experiment on an etherized duck 310
Methods 315
Conclusions 316
Note 316
Bibliography 317

INTRODUCTORY

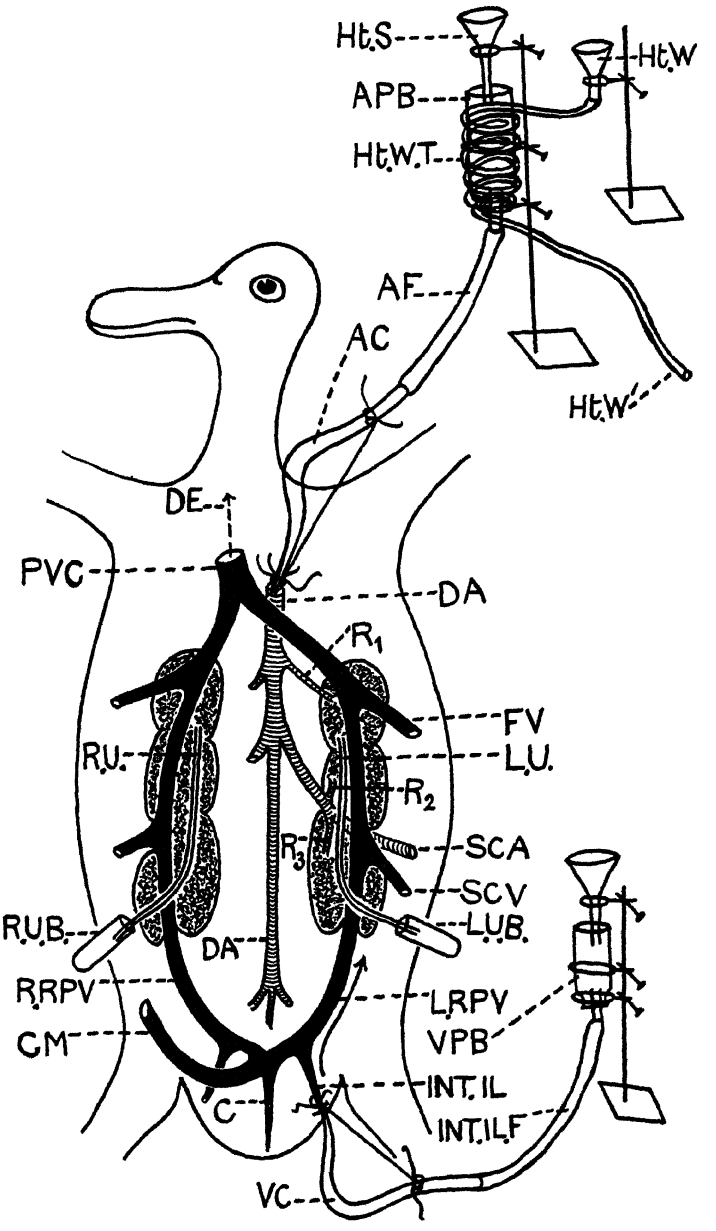
Some time ago, while working out the general disposition and anatomical relations of the principal veins in connection with the kidneys of certain common Indian birds (Das, '24), it seemed to me that it might be worth while to try an experiment of a physiological character by allowing a certain fluid containing dye to pass through the 'renal-portal' vein (renal afferent) in order to demonstrate the direction of the current of the perfusing fluid (and hence that of blood in nature) as

well as to investigate if the 'renal-portal' vein gives out fine capillaries in the renal tissue or not; in other words, I wished to ascertain if, under conditions of pressure maintained approximately the same as those found in nature, 1) the current in the renal afferent (or the 'renal-portal') vein was directed anteriorly or posteriorly, and, 2) if anteriorly, whether or not the fluid in the renal afferent vein penetrated to any extent into the smaller channels (sinusoids or capillaries) of the kidney substance in birds.

MODUS OPERANDI AND EXPERIMENT ON AN ETHERIZED DUCK

For this purpose a male domesticated duck (of the genus Anas) was deeply anaesthetized with ether, and kept in this condition while the body cavity was opened up posteriorly to the keel of the sternum. The coeliac and anterior mesenteric arteries were ligatured, and the aorta (*DA*, fig. 1) cut through about 2 cm. anteriorly to the ligatured coeliac artery. The kidneys and the ureters were also exposed and all kidney veins. The other viscera were kept intact, and were displaced to the left side for the sake of convenience of the experiment. Each ureter (*R.U.* and *L.U.*) was cut through posteriorly, cleared from connective tissue, and its cut

Fig. 1 Diagrammatic ventral view of a male duck. *AC*, aortic cannula; *AF*, arterial perfusion rubber-tubing connection; *APB*, arterial perfusion bottle containing 9 per cent saline, to every 100 cc. of which 0.005 gram of uric acid and 2 cc. of normal human urine have been added, and the fluid kept at a pressure of 60 cm. and maintained at a temperature of 44°C.; *C*, caudal vein; *CM*, coccygeomesenteric vein; *DA*, dorsal aorta; *DE*, dye exit; *FV*, femoral vein; *Ht.S*, funnel to carry the fluid for arterial perfusion; *Ht.W*, funnel to carry hot water circulating outside the arterial perfusion bottle; *Ht.W.'*, hot-water waste; *Ht.Wt.*, rubber tubing spirally coiled outside the arterial perfusion bottle and carrying hot water so as to keep the bottle warm; *INT.IL*, left internal iliac vein; *INT.IL.F*, 'renal portal' perfusion rubber-tubing connection; *L.RPV*, left 'renal-portal' vein; *L.U.*, left ureter; *L.U.B.*, left ureter bottle; *PVC*, posterior vena cava; R_1, R_2, R_3, renal arteries; *R.RPV*, right 'renal-portal' vein; *R.U.*, right ureter; *R.U.B.*, right ureter bottle; *SCA*, sciatic artery; *SCV*, sciatic vein; *VC*, 'renal-portal' cannula; *VPB*, 'renal-portal' (venous or internal iliac) perfusion bottle containing at first the same fluid as the bottle *APB*, and kept at 5-cm. pressure and at 60°C. in the bottle. The arrowhead shows the direction of the perfusion current. The figure is not drawn to any definite scale; it is merely schematic.


Ht.S
APB
Ht.W.T
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AC
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FV
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L.U.
R2
R3
SCA
SCV
R.U.B.
L.U.B.
DA
R.R.P.V.
L.R.P.V.
C.M.
VPB
INT.IL
C
INT.IL.F
VC

Figure 1

extremity inserted into the mouth of a glass tube (*R.U.B.* and *L.U.B.*, respectively) supported on putty. Into the cut end of the aorta was inserted a cannula (*AC*) connected with an arterial perfusion bottle (*APB*), containing 9 per cent saline solution, to each 100 cc. of which had been added 0.005 gram of uric acid (only slightly dissolved) and 2 cc. of normal human urine, at a pressure of 60 cm. and maintained at a temperature of 44°C. in the perfusion bottle (by means of hot water circulated through a rubber tubing (*Ht.W.T*) surrounding the perfusion bottle). Into the left internal iliac vein (*Int.Il*) was inserted another cannula, viz., the 'renal-portal' (*VC*) with a much smaller nozzle (and, therefore, having very low rate of flow as compared with the aortic cannula, *AC*) connected with a second perfusion bottle (*VPB*), at first containing the same fluid at a pressure of 5 cm. (raised temporarily to 7 cm. during the experiment) and at a temperature of 60°C. in the bottle. In a previous experiment on another duck, under the same condition, the arterial flow, measured for half an hour, was 2063 cc. and the left internal iliac vein flow was 135 cc., giving flows, respectively, of 343.7 cc. and 22.5 cc. for five minutes, hence it cannot be supposed that the renal afferent (i.e., the venous) flow was excessive relative to the arterial. The experiment was set going at 10.45 A.M. and after thirty-five minutes (i.e., at 11.20 A.M.), the internal iliac perfusion bottle (*VPB*) was quickly filled with warm carmine solution (carmine powdered and filtered in the perfusion fluid through fine-meshed copper gauze) which entered the left internal iliac vein via the cannula (*VC*) at 11.47 A.M. (internal iliac perfusion bottle at 7-cm. pressure now). I could easily trace the path of the dye, which flowed slowly forward into the left renal afferent ('renal-portal') vein, and thence into the postcaval vein. En route, the substance of the posterior lobe of the left kidney[1] became partially tinged with red (apparently, of course, due to a network of channels much larger than the capillaries), and the middle and the anterior lobes were just slightly

[1] See Note.

tinged in places. The coccygeomesenteric contained no dye. At 11.51 the internal iliac bottle was lowered to 5-cm. pressure to see if the dye could be removed from the kidney substance, but no change occurred in this respect.

These results certainly indicate that the blood in nature flows anteriorly in the renal afferent ('renal-portal') vein, but I am not quite certain that the penetration of the carmine into the smaller channels (evidently not capillaries) of the posterior lobe of the kidney indicated that the venous blood in nature also penetrates into these smaller channels, because although the pressure in, and therefore the flow from, the internal iliac perfusion bottle cannot be supposed to have been excessive, yet it is not impossible that the dye merely diffused against such a slow current, and therefore gave erroneous impression of the renal afferent vein fluid penetrating into spaces which were in reality affluent and not effluent.

During the whole experiment and previous to the experiment the kidneys were in a state of great activity. Before the bird was completely etherized, semisolid, cream-colored uric acid could be seen traveling down the ureter at a great rate. Even after the perfusion fluid had been substituted for the blood, the urine still maintained its semisolid character, at least for some time. At 10.50 the two ureters (*L.U.* and *R.U.*) were inserted into tubes (*L.U.B.* and *R.U.B.*), and the first left[2] tube (i.e., the one for the right ureter, *R.U.*) was removed at 11 A.M. and another one substituted. At 11.20 the first right[2] tube (i.e., the one for the left ureter, *L.U.*) and the second left tube (i.e., the right ureter) were removed. The first left tube contained 1.7 cc. of urine, resembling milk in appearance; the second left tube contained 1.2 cc., more liquid in appearance (turbid); the first right tube contained 2.4 cc. of urine, milky in appearance, but less so than that contained in the first left tube. At 11.20 fresh tubes were placed. The third left tube (i.e., the right ureter) was

[2] The terms 'left' and 'right' refer to the author's left and right, and not the animal's.

removed at 11.47, and contained about one drop of watery fluid; at 11.57 the second right tube (left ureter) was removed, and contained 1.15 cc. of watery fluid (no uric-acid crystals visible).

Since even when the aorta was perfused with the perfusion fluid the first lot of urine secreted was still milky in appearance and, in fact, strongly resembled the natural avian urine secreted previous to the experiment (though naturally somewhat more liquid), it would appear probable from this single observation that the kidney itself secretes the semisolid urine characteristic of birds, and that the statement, quoted by Cushny (from Sharpe), that the urine of birds as formed by the kidneys is liquid like that of mammals while traveling down the ureters, and only assumes its semisolid character by subsequent absorption of water in the cloaca, is erroneous. I may also add that I have frequently observed solid uric acid in the ureters of about 200 birds that I have so far dissected.

From these experimental results it follows that the direction of blood in the 'renal-portal' veins of birds is anterior, and that the 'renal-portal' vein does not give out fine capillaries in the kidney substance as in fishes, amphibians, and reptiles; the latter assumption is also strengthened by actual measurements of the calibers of the renal afferent veins at their various lengths. Each 'renal-portal' vein in its course through the kidney substance postero-anteriorly distinctly increases in caliber. Thus in one pigeon (Columba intermedia) examined the internal circumference of the right renal afferent vein posteriorly (where it first entered the substance of the posterior lobe of the kidney) was 2.61 mm., giving an area in transverse section of 0.543 sq.mm., while anteriorly (immediately previous to its junction with the femoral vein) the internal circumference was 3.18 mm., giving an area in transverse section of 0.805 sq.mm.; the internal circumference of the left renal afferent vein posteriorly was 3.32 mm., giving an area in transverse section of 0.878 sq.mm.; while anteriorly it was 3.5 mm., giving an area in transverse

section of 0.975 sq.mm. Similar results have also been obtained from the measurements taken in the case of the domesticated duck. In the duck the internal circumference of the right renal afferent vein as measured posteriorly was 7.3 mm., thus giving an area in transverse section of 4.243 sq.mm., while anteriorly it was 6.8 mm., thus giving an area in transverse section of 3.682 sq.mm.; the internal circumference of the left renal afferent vein posteriorly was found to be 6.7 mm., thus giving an area in transverse section of 3.574 sq.mm.; while anteriorly it measured 7.2 mm., thus giving an area in transverse section of 4.127 sq.mm. Thus the two 'renal-portal' veins are unequal in their calibers throughout their course, and evidently increase in capacity as they pass from behind forward.

METHODS

For the measurements of the calibers of the 'renal-portal' veins a pigeon and a duck were slightly opened up from the ventral side and preserved whole in 6 per cent formalin for a week. The 'renal-portal' veins were afterward fully exposed under a binocular, and small lengths of them from the posterior and anterior ends were removed and embedded in paraffin, and serial sections were mounted on the slide. The sections were stained with borax-carmine and then differentiated with picro-indigo-carmine. The internal circumferences were drawn by means of camera lucida under magnifications of about 171 diameters for the pigeon and about 76.7 diameters for the duck. The circumference was measured in each case by means of a string passed exactly round every curvature, and their areas were then calculated and compared.[3]

I am extremely grateful to Dr. W. N. F. Woodland for all the kind help and suggestions he has given me in connection with this work.

[3] This is more or less a fair estimate, and gives at least some idea of the calibers of the vessels in question.

CONCLUSIONS

1. The current of blood in the 'renal-portal' veins of birds is *anterior.*

2. The 'renal-portal' veins *do not* break up into fine *capillaries* or *sinusoids* as in fishes, amphibians, and reptiles, but pour their blood *directly* into the posterior vena cava with undiminished caliber. In other words, *there is no possibility of a 'renal-portal' system in birds.*

3. The urine of birds is *semisolid,* and *not* liquid, and there is hardly any absorption of water in the cloaca.

NOTE

I noticed that the dye tinged the substance of the kidney in parts only, and it therefore does not signify that the dye actually entered into the fine renal capillaries. If such had been the case, the renal tissue as a whole should have taken on a uniformly deep red stain all over. The mere fact that the kidney substance was touched *in parts* (and especially its posterior part) affords a strong evidence in favor of the view that there had probably been a slow process of physical diffusion of the dye (i.e., the apparent entry of the dye in places which, as a matter of fact, should not have normally taken place) in the venous channels (and not in fine capillaries, of course) in which blood in life really flows toward and not away from the renal afferent vein. This statement is further strengthened by the fact that the diameter of the renal afferent vein (right and left) at the anterior end of the kidneys (examined in the duck and the pigeon) is greater than at the posterior end, which implies that it must have been fed by venous tributaries (i.e., affluents) during its passage through the kidney substance, and that it does not give out branches (i.e., the effluents) which would ultimately capillarize, because if the latter condition exists, it would be quite reasonable to expect the diameter of the vein at the anterior part of the kidney to have become decidedly smaller as compared with that at the posterior end, but this is exactly contrary to the observed facts—*it actually gains in caliber from behind forward gradually as it proceeds toward the anterior end, as shown by measurements given in the text.* The presence of numerous small renal veins (i.e., the venous channels or affluents just referred to, in addition to the large principal renal efferent vein), which pour their blood into the renal afferent vein (this latter apparently being nothing more than a big reservoir of blood as it courses through the substance of the kidney), is probably correlated with the increased functional activities in Aves.

Cushny ('17, p. 53) states that "A more cogent argument is the absorption of urine itself in the Birds; in these the urine passes down the ureter as an abundant and generally *clear* fluid, but in the bowel the water is almost absorbed leaving a thick paste of urate" (Sharpe, '12; compare Das, '24, p. 771).

In order to verify Cushny's remarks, I vivisected a large number of Indian birds, and I found that in each and every one of them, without exception, the ureter from the very beginning was full of semisolid cream-colored urine. I never found any visible trace of watery fluid under natural conditions in any part of the ureter; the wall of the ureter was sufficiently thin so as to allow the semisolid matter to be seen along its whole length, and the kidneys were actually seen to be actively secreting this semisolid urine all the time.

Furthermore, on opening any etherized bird it will be at once seen that the semisolid urine passes down the ureter by a peculiar slow peristaltic movement of the muscular walls of the ureter, and on reaching the cloaca it is voided along with the faecal matter. It may be stated from experimental observations already recorded that the kidneys, even long after being perfused with the saline mixture, secreted highly milky urine, which gradually took the form of a watery fluid when all the solid urates were practically washed down, so to speak, during perfusion. This conclusively proves (and similar features having already been noticed in some 200 specimens that I have dissected) that *in birds the urine is always and from the very start solid,* and *not* watery as in other animals; and this fact is probably correlated with the greater activity in birds as compared with other animals, and hence the necessity for the conservation of water (circulating in the blood) by the renal cells.

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BLOOD FORMATION IN THE AFRICAN LUNGFISH, UNDER NORMAL CONDITIONS AND UNDER CONDITIONS OF PROLONGED ESTIVATION AND RECOVERY[1]

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FOUR PLATES (ONE HUNDRED FIGURES)

AUTHORS' ABSTRACT

The blood cells of the African lungfish, Protopterus ethiopicus, are very large and resemble those of urodeles. Leucocytes are especially plentiful and rich in variety, including eosinophils, special eosinophils, 'meta-eosinophils' with atypical granules, monocytes, thrombocytes, lymphocytes, and basophils.

The chief hemocytopoietic organs are the spleen, intestine, and kidneys. The lungfish spleen, embedded in the wall of the stomach, represents an intermediate phylogenetic stage between the disperse intra-enteral type of the hagfish and the compact extra-enteral type of other vertebrates.

Erythrocytes are formed in the spleen pulp, granulocytes in the granulocytopoietic organ of the intestine and in the capsules of kidneys, gonads, and spleen. Thrombocytes and monocytes are differentiated in the spleen and general circulation. Basophils arise in the spleen and intestine. Lymphoid cells of all types arise in the spleen. Evidence is presented bearing upon the hemocytopoietic capacity of the various types. Cells with 'Russell bodies' also occur in the spleen.

In lungfishes subjected to long periods of dry estivation, erythrocytopoiesis practically ceases. Granulocytes, however, appear to play an important rôle, possibly in fat metabolism. The large variety of meta-eosinophils, a unique feature of the lungfish, appears to be associated with the habit of estivation.

Recovery from estivation may show numerous amitoses of erythrocytes in the general circulation. Other cells which divide in this manner are young thrombocytes, granulocytes, monocytes, and lymphoid hemoblasts.

CONTENTS

Introduction 320
Material 321
Observations 322
The general circulation 322
Erythrocytes 322
Amitosis of erythrocytes 323
Senile erythrocytes 324
Erythroplastids 325
Leucocytes 325
Lymphoid hemoblasts 325
Small lymphocytes 325
Thrombocytes 326
Mitosis and amitosis 326

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Monocytes and macrophages 327
Eosinophilic granulocytes 328
Basophilic granulocytes 328
The hemocytopoietic organs 329
Spleen 329
Central lymphoid mass. Lymphocytopoiesis 330
The splenic pulp region. Erythrocytopoiesis 332
Thrombocytopoiesis 333
Monocytopoiesis 334
Granulocytopoiesis 334
Eosinophils 334
Special eosinophils 335
Meta-eosinophils 336
Basophils 336
Disposal of granulocyte débris 337
Disposal of erythrocyte débris 338
Observations upon various organs, including kidneys, gonads, liver, heart, stomach, bursa entiana, pancreas, lungs, vertebral column, muscle, and fat 338
Estivation 340
Comparison of hemocytopoietic organs of lungfish fry, young lungfish, and adult 343
Discussion 345
Hemocytopoietic loci and the phylogeny of the spleen 345
Lymphocyte types and their hemocytopoietic capacity 346
Seasonal and regional activity of the spleen 348
Significance of erythrocyte amitosis 349
The granulocytes; senility and relation to pigment formation 351
Granulocytes and fat metabolism 352
Monocytes and macrophages 354
Thrombocytes 354
Double origin of pigment cells 356
Summary 356
Literature cited 357

INTRODUCTION

The lungfishes constitute an especially interesting group from the hemocytopoietic standpoint. There has, however, been no recent or detailed work on their blood and blood-forming organs. These animals, being native to Africa, South America, and Australia, are not readily available for study and experiment.

Preliminary observations revealed that both erythrocytes and leucocytes are of exceptionally large size, being comparable in this respect to the blood cells of urodeles. Parker

('92) in his monograph on the African lungfish remarks that this animal is probably richer in leucocytes than any other vertebrate. Not only are the leucocytes relatively numerous, but they exhibit an extraordinarily wide variety of types. Another interesting characteristic of the lungfish is its habit of existing in a torpid condition for months in moist or even dry earth after the evaporation of the water in which it is living. That such estivation should have a profound effect on the hemocytopoietic activities seemed most probable.

Through the courtesy of Prof. H. W. Smith, of New York University, material of the African lungfish, Protopterus ethiopicus, was obtained. This included normal young and adult animals, as well as animals subjected by Doctor Smith to various degrees and lengths of estivation.

MATERIAL

Material from the animals listed below was available for this study.

No. 1. Young lungfish, 14 cm. in length, about two years old, collected in Africa during the latter part of July, 1928, placed in a small can of slightly moist earth for two months (torpid condition), then in water for five days (active condition), and killed.

No. 2. Adult lungfish, about 60 cm. in length, possibly six or more years old, taken from the lake in Africa and killed immediately, July 28th.

No. 3. Several small lungfish fry, 2.5 cm. in length, about two months old, collected in July and killed immediately.

No. 8. Lungfish collected about middle of July, no food given, kept alive in dry estivation from November, 1928, to October, 1929, killed without being returned to water.

No. 13. Lungfish collected in July, kept alive in dry estivation from November 1, 1928, for 410 days, placed in water for thirteen days, infection appearing. Died during night and tissues fixed next morning. During the last two days of life, treatment with potassium permanganate was resorted to in an attempt to overcome the infection.

No. 21. Lungfish collected in July, kept alive in dry estivation from November 1, 1928, for 427 days; replaced in water for ten days and killed, as it seemed about to die after the appearance of superficial infection. An injection of thyroxin (0.5 mg.) twenty-one days before death resulted in 100 per cent rise in metabolic rate. Original weight, 182 grams; dry estivation weight, 133 grams; postestivation weight after water absorption, 152 grams.

No. 29. Lungfish collected in July, subjected to dry estivation from November 1, 1928, to January 21, 1929, being kept at 90°F. for three weeks and 103° F. for four days; placed in water January 21, 1929, died on fourth day, an infection being present.

Nos. 51 to 70. Twenty lungfishes fixed in 10 per cent formalin, varying in size from 15 cm. to 35 cm., thought to be from two to four years old.

No. 33. Lungfish collected in July, 1928, kept in water until January 10, 1930, being fed regularly; killed by cold in transportation between Detroit and New York, tissues, however, showing little post-mortem change.

No. 34. Lungfish with history like that of no. 33.

Blood smears were available for study from animals nos. 1, 8, 13, 21, 33, 34. These were stained according to the Wright technic. The tissues were fixed in the Zenker-formol mixture of Helly and stained with eosin-azure mixture of Giemsa, or with eosin-azure following dilute Delafield hematoxylin after the method of Maximow.

The regions which are of chief interest for the study of hemocytopoietic activity are the following:

1. The general circulation for the variety of types of blood cells.

2. The spleen for the processes of lymphocytopoiesis, erythrocytopoiesis, granulocytopoiesis, erythrocytophagic and granulocytophagic activity, and for its place in the general scheme of spleen phylogeny.

3. The spiral-valve region of the intestine for its granulocytopoietic organ.

4. The kidney and gonad capsules for granulocytopoiesis.

OBSERVATIONS

The general circulation

Erythrocytes. Mature, young, and senile erythrocytes are present in the general circulation (figs. 1 to 6). The resemblance to conditions in the salamander is striking. The ancestral cell is the lymphoid hemoblast (hemocytoblast). An early stage in erythrocytopoiesis is represented by the proerythroblast (fig. 1). The nucleus in this stage is often somewhat condensed, granular, and tachychromatic, the nuclear membrane sharply defined, and the cytoplasm deeply stained,

with sometimes a non-homogeneous appearance. Irregular outline of the border of the cell may also characterize this stage. While the pro-erythroblast figured (fig. 1) is of large size, many cells at this stage are much smaller and should not be confused with cells of the lymphoid hemoblast group. The pro-erythroblast nucleus always presents a dark blue color as compared with the purple or light blue of the ancestral lymphoid hemoblast. Similar conditions have already been described in detail for salamanders (Jordan and Speidel, '30). Numerous transitional forms may be found between the pro-erythroblast stage and the adult erythrocytes (figs. 2 to 4). Progressive increase in hemoglobin gives the characteristic staining reactions of the cytoplasm. The nucleus loses its somewhat granulated appearance and large deeply stained chromatin blocks are formed, with a tendency to thread formation. In the final stages contraction of the nuclear membrane may leave these projecting somewhat at the border. The shape, both of nucleus and cytoplasm, changes from spherical to oval.

Mitoses of erythroblasts are fairly common in the blood smears of normal animals (nos. 33, 34). They are not to be seen in the estivating specimens. In the splenic sinuses of animal no. 2, freshly taken from the lake in Africa, numerous mitoses of hemoblasts and of all stages in erythrocyte differentiation are present (fig. 37). Blood smears of this specimen were not, however, available.

Amitosis of erythrocytes. In an animal (no. 1) which was placed in water for five days after two months of estivation, amitoses of erythrocytes are numerous (fig. 16). Though some of these cells appear to be not entirely mature, others seem to have their full complement of hemoglobin.

In a few cells, definite cell plates between the daughter cells can be recognized—an observation which we believe precludes the possibility of the amitotic appearance being the result of artifacts. Very early stages in the process can be recognized under high magnification (figs. 93, 94). The first indication is a slight thinning of the nucleus near the center and the

appearance of a fairly definite 'nuclear plate' (figs. 93, 94). A slight indentation at the edge of the nucleus is also usually present (fig. 93). The cytoplasm also shows elongation and central constriction (figs. 94 to 97). As the nuclear halves draw apart, fine lines of chromatin usually still connect the two halves (figs. 94 to 97 and 16). Although in a few cases a sharp cell plate can be noted (fig. 16), the more usual condition shows the central region reduced to a fine thread-like connection (fig. 97).

A random count under high magnification of 10,000 erythrocytes in animal no. 1 showed fourteen indubitable cases of amitosis and thirteen other probable cases (early stages). In some low-power fields three or four examples were to be seen. That the amitotic impulse does not always result in nuclear division is suggested by the cell of figure 98. We are inclined to interpret this as a case of cytoplasmic segmentation—a process which is common to many urodeles, but which is quite rare in lungfish. It is quite obvious that the daughter halves in amitosis are practically always of distinctly different size, the discrepancy often being considerable. Amitoses of erythrocytes occur in some of the lungfishes other than no. 1, but they are much less numerous.

Senile erythrocytes. The most usual type of senile erythrocyte presents a pale shadowy cytoplasmic envelope and swollen, lightly staining, purple nucleus. At a later stage the cytoplasm disappears entirely and the nucleus becomes progressively less definite.

Another type of senile cell has the 'soap-bubble' type of nucleus (fig. 5). In this type the nucleus is vacuolated and the nuclear membrane distended in an irregular manner, taking on a lobulated appearance. As degeneration proceeds the nucleus progressively stains more green and less blue. Many degenerating erythrocytes of this type are present in our estivating animals. We have noted this type of degeneration in salamanders (Jordan and Speidel, '30) and it has recently been described in some detail in infected Triturus (Nigrelli, '29).

A third type of erythrocyte degeneration occurs occasionally, a type which may be termed 'intracellular nuclear fragmentation' (fig. 6). The chromatin material of the nucleus is scattered throughout the cell. Both cytoplasm and the chromatin masses often show degenerative vacuolation. In some erythrocytes a densely pycnotic nucleus occurs with or without vacuoles.

Erythroplastids. Erythroplastids are exceedingly scarce, though there is evidence of occasional cytoplasmic segmentation (fig. 98).

Leucocytes. Leucocytes are especially numerous in proportion to erythrocytes in the lungfish blood smears. Many types occur, including lymphoid hemoblasts (large lymphocytes), small lymphocytes, thrombocytes, eosinophilic granulocytes, special eosinophilic granulocytes, meta-eosinophilic granulocytes, monocytes, macrophages, basophilic granulocytes, and lymphoid cells with acidophilic inclusions.

Lymphoid hemoblasts (large lymphocytes). The lymphoid hemoblasts are large lymphocyte-like cells (figs. 7, 11, 17). The nucleus contains a moderate number of fair-sized chromatin blocks and varies in staining reaction from blue to purple. Plasmosomes, though conspicuous in fixed material, do not show in the smears. The light blue cytoplasm is homogeneous, or slightly granulated, with weakly basophilic (bluish) granules. Pseudopods may be present. That some of these cells undergo degeneration is clear. Transitional forms with lightly staining nucleus and cytoplasm, followed by typical degenerating stages, suggest that many of these cells never give rise to anything other than a lymphoid cell. Other transition stages, however, seem to point to this type of cell as the progenitor of the large thrombocyte and monocyte as well as erythrocyte.

Small lymphocytes. Small lymphocytes occur in moderate numbers. They present a number of chromatin blocks in the nucleus which stain relatively deeply. The cytoplasm is usually somewhat granular. In many cases the nuclei are practically naked, with the thin cytoplasmic rim reduced to a

minimum. The nucleus may be slightly grooved or lobulated. A few basophilic or azurophilic granules may be present. Often lymphocytes with little or no cytoplasm so closely resemble thrombocytes which have also lost their cytoplasm that a distinction cannot be definitely made. Of special interest is the occasional occurrence of large eosinophilic inclusions in small or medium-sized lymphocytes (fig. 22).

Thrombocytes. The thrombocyte is characterized by a small condensed nucleus and very little cytoplasm, which usually presents some reddish or reddish-violet granulation (fig. 14). The nucleus is oval or spherical, and stains deeply. Grooving of the nucleus may be observed in many cases. The delicate pale-staining outer rim of cytoplasm is not always conspicuous, but may be seen in thrombocytes that have clumped (fig. 14).

Stages in the development of thrombocytes occur in the general circulation. The youngest thromboblasts resemble the medium-sized or small lymphoid hemoblasts (figs. 12, 13). The nucleus is slightly more dense. The cytoplasm is characterized by mixed granules, both bluish and reddish ones being conspicuous. At a later stage the bluish granules disappear and are replaced by reddish ones. The outer portion of the cytoplasm becomes rather delicate and often shows processes. The granules may be present all around the nucleus, but there is a tendency for segregation at one or both poles. In later stages the nucleus becomes still more dense and the cytoplasm more scanty.

Mitosis and amitosis. One interesting example was seen of a thromboblast in mitosis in the circulation. The cell was in early prophase, and the cytoplasmic granules were mixed, both blue and red ones being visible. We have previously recorded two cases of thromboblast mitosis in the circulating blood of splenectomized salamanders (Jordan and Speidel, '30).

Another observation showed that thromboblasts may divide by amitosis (fig. 15). Several other cases of probable amitosis of young thrombocytes were seen. These observations

were made on the blood of a young lungfish (no. 1) placed in water for five days after being in slightly moist earth for two months. This is the same animal which displayed such a large number of erythrocytes dividing by amitosis.

Monocytes and macrophages. Typical monocytes are present in fairly large numbers (figs. 8, 9). The nucleus may be reniform as figured, or it may be spherical, elongate, lobulated, or double. It presents a purplish tinge in Wright's stain, or in some preparations a light blue color. The chromatin blocks often have a 'smeared' appearance. The cytoplasm may be characterized by a mixed weak reddish and bluish granulation. An archoplasmic area may be easily seen. There is a distinct tendency for the reddish granules to be aggregated about this area (fig. 8). However, the reddish granulation may be entirely absent. The contour of the cell may be smooth or it may show irregular processes. Great size variation has been noted. In general, the monocyte is of a size comparable to the large lymphoid hemoblast and often it is somewhat larger. However, a few small cells have been seen possessing distinct monocyte characteristics.

Monoblast stages have been seen which are transitional between the hemoblast (fig. 7) and the monocyte (fig. 8). A young monocyte has a spherical nucleus and often a mixed blue and red granulation. Some cells of this type approach in appearance the thromboblasts, so that it is not always easy to distinguish between the two.

The cytoplasm of the monocyte may show vacuoles as well as granules and present a 'stringy' appearance (fig. 9), similar to conditions in the salamander. Cells of this type may become quite active in phagocytosis. One of these is illustrated (fig. 10). Among the ingested materials of these monocytic macrophages may be recognized much eosinophil débris.

Several examples quite suggestive of multiplication of monocytes by amitosis have been seen. Both monocytes and macrophages are quite numerous in animals no. 21, 13, and 8. Whether this is a result of estivation, or of infection, or both is not certain.

Eosinophilic granulocytes. Large eosinophils with coarse granules are conspicuous (fig. 18). The nucleus is often approximately spherical or slightly indented, but it may also be lobular, or even occur in two or three separate parts. An archoplasmic area devoid of granules is visible. The cytoplasmic ground-substance stains a pale bluish gray. Variations in size are marked. Plastids of these cells may be seen occasionally. Rarely, eosinophils with pigment granules occur in the general circulation. Similar pigmented eosinophils have been seen in the circulating blood of the salamander.

Coarse-granule eosinophils in all stages of senility and degeneration may be found. These are particularly in evidence in animals subjected to long periods of estivation. There is often coalescence of the granules into large masses which then may lose their eosinophily. Cells sometimes appear to break into several parts. Another type of degeneration is characterized by a progressive lightening of staining reaction, both of granules and nucleus. In some cases pigment granules are produced as a late stage of the degenerative process.

Special eosinophilic granulocytes. Eosinophils with fine granules are numerous (fig. 19). The nucleus is ovoid, sometimes reniform, lobular, ring-like, or double. An archoplasmic area may, or may not, be visible. The cytoplasmic ground-substance stains a pale bluish gray.

Degenerative forms of this type of cell are frequently met with, especially in animals subjected to estivation. Degeneration usually causes the cell to stain with a pale watery appearance, the nucleus and granules losing their distinctness (fig. 20).

Basophilic granulocytes. Basophils with metachromatic granules (lilac or violet) occur abundantly (fig. 21). The nucleus is usually approximately spherical. It may, however, be bilobed or grooved. The granules may be coarse or fine. The cells often present a somewhat degenerate appearance, with nuclear structures indistinct. Some cells seem to show

liquefaction and disappearance of the granules. Young stages of basophils may also be seen which contain a single row of metachromatic granules and a young-looking nucleus. In these young cells may sometimes be seen the weakly bluish granules characteristic of the lymphoid hemoblast, which is considered the progenitor.

The hemocytopoietic organs

Spleen. The spleen of the lungfish is the chief organ for erythrocytopoiesis. It is in close relation to the stomach, being embedded in the wall and covered by peritoneum and muscle (figs. 47, 48). The pancreas is immediately beside the spleen and has a similar relation to the stomach (fig. 48).

In the young lungfish (fig. 48) the spleen presents a central core of lymphoid cells, surrounded by a region of pulp cords and sinuses, and a thin peripheral capsular region. Connective-tissue strands extend at intervals between the outer capsule and the central lymphoid mass, thus dividing the pulp into lobular areas. From the central lymphoid core lobular projections of lymphoid cells extend into the pulp areas. The spleen of the older lungfish (60 cm. in length) resembles that of the younger animal except that with the increase in size there has also gone increase in number of lobules of splenic pulp, as well as increase in the lobulation of the central lymphoid mass. Some of the lobules of lymphoid tissue appear to have become separated from the central mass. A study of each of these regions in the spleen discloses characteristic functions.

In the central lymphoid mass lymphocytopoiesis is actively carried on. In the splenic pulp erythrocytopoiesis takes place. Senile granulocytes and erythrocytes are disposed of by macrophages, reticular cells, and endothelial cells in this region. A certain amount of extravascular granulocytopoiesis and intravascular thrombocytopoiesis may also be seen. In the capsular region granulocyte production and destruction may both be active. The latter process is marked by extensive pigmented areas of degeneration.

The central lymphoid mass. Lymphocytopoiesis. In the central lymphoid mass there are present lymphocytes of large, medium, and small size, the last type predominating. Reticular cells form the framework, and there are small arteries at intervals, but no veins. The lymphocytes migrate peripherally into the splenic pulp.

The larger and medium-sized lymphocytes usually present the typical vesicular or 'sieve' type of nucleus, the chromatin being in the form of a few large angular blocks and fine granules scattered through the linin. One or more plasmosomes may be present, staining red with the eosin-azure combination (figs. 52, 56). This type appears to be the usual hemoblast, the progenitor of erythrocytes, monocytes, and granulocytes. It is identical with the hemoblast type found in the granulocytopoietic organ of the intestine (fig. 23), which in that locus gives rise to granulocytes. In some of the large and medium-sized lymphocytes the large chromatin blocks may be numerous and radially arranged (figs. 55, 59), somewhat resembling, therefore, the small lymphocyte type. The small lymphocytes (fig. 63) usually possess a moderately condensed nucleus with large angular chromatin blocks arranged in more or less radial fashion (wheel arrangement). Plasmosomes are usually not present, though in some cases they may be seen. They are not as conspicuous as in the larger lymphocytes. A few small-sized lymphocytes with nuclei of the vesicular type may be found (fig. 60), as well as intermediate types (figs. 61, 62).

There are, therefore, lymphoid cell types (figs. 52 to 63) which may be classified as follows: 1) Large lymphocytes with *a*) amblychromatic nucleus (fig. 52), *b*) pachychromatic nucleus (fig. 55) with radial arrangement of chromatin blocks ('cartwheel' type), *c*) intermediate nuclei between *a* and *b* types (figs. 53, 54). 2) Medium-sized lymphocytes with *a*, *b*, and *c* types of nucleus (figs. 56 to 59). 3) Small lymphocytes with *a*, *b*, and *c* types of nucleus (figs. 60 to 63). Types 1*c* and 3*a* are least numerous. The large number of transitional lymphocytes with respect to size, arrangement of

chromatin, amount of chromatin, and occurrence of plasmosome suggest that all of the lymphoid cell varieties are genetically related. Mitoses of medium-sized and large lymphocytes are common. Small lymphocytes, however, seem not to multiply in this fashion. On the contrary, the large number of deeply lobulated and even binucleated forms indicates the probability that some multiplication of small lymphocytes by amitosis occurs (figs. 87 to 89).

The smaller lymphocytes with coarse chromatin granules in the nucleus are especially conspicuous near the periphery of the lymphoid core. They appear to arise from the less differentiated lymphoid cells nearer the center, and to migrate into the splenic pulp. Some undergo degeneration (figs. 78 to 80) and some remain as lymphocytes in the general circulation. This, however, accounts for only a portion of them. Many, after reaching the venous sinuses of the spleen, function as the progenitors of thrombocytes.

The reticular cells of the lymphoid core present nuclei much like those of the large lymphocytes (figs. 81, 82). The chromatin is sparse and in the form of fine granules except for a few larger blocks. A plasmosome is usually distinguishable. The boundary of the irregular cytoplasm cannot ordinarily be traced exactly, as it extends out in interlacing processes. In some of the cells a faint cytoplasmic granulation may be made out.

The close resemblance of the nucleus of a reticular cell to the vesicular sieve-like nucleus of many of the lymphocytes (fig. 83) makes it seem probable that some reticular cells by rounding up may become transformed into lymphoid hemoblasts. Many reticular cells contain granules (fig. 84). This condition is especially noticeable in regions from which most of the lymphoid cell content is gone ('exhausted' areas) and in reticular cells of the splenic pulp. In many cases these granules appear to be derived from senile granulocytes and ingested by reticular cells. However, in other cases the granules appear to be a product of the reticular cells.

The splenic pulp region. Erythrocytopoiesis. The most striking feature of the spleen pulp is the large number of pro-erythroblasts and erythroblasts. These cells are conspicuous in the venous sinuses of this region and in the meshes of the pulp cords. The youngest stages of development of these cells plainly indicate the usual mother cell. It is the lymphoid hemoblast of large or medium size with vesicular, sieve-like nucleus. Stages in the differentiation of three kinds of lymphoid hemoblasts into erythroblasts are shown (figs. 64 to 75); in one row a medium-sized vesicular hemoblast with the amblychromatic nucleus persisting well into the erythroblast stage (figs. 64 to 67); in the next row a typical 'intermediate' type of hemoblast with pro-erythroblast and erythroblast stages following (figs. 68 to 71); and in the next row, a hemoblast with lobulated nucleus followed by pro-erythroblasts and erythroblast also with lobulated nucleus (figs. 72 to 75). The pro-erythroblast stage may be recognized by a slight change in the staining reaction of the cytoplasm from the gray-blue of the hemoblast to a slightly more purple tinge. The nucleus becomes less sieve-like; i.e., the fine chromatin granules on the linin are no longer so obvious. The blocks of chromatin become less angular and in some cases slightly swollen, in others slightly elongated. These chromatin blocks stain less deeply blue and less sharply. The plasmosome persists, but is often slightly less conspicuous.

The erythroblast is characterized by pronounced hemoglobin formation resulting in a definite pinkish-staining reaction. The nucleus may show some contraction, but the early erythroblast nucleus may be as large as that of the hemocytoblast ancestor. The sieve appearance of the earlier stages is usually practically gone, especially in the later erythroblasts. The chromatin blocks are still less angular. They are more numerous and in many cases show a tendency to elongate and coalesce with adjacent granules into threads. Plasmosomes are still visible, though often not very obvious (figs. 36, 38). In some cells there is exhibited a tendency on the part of the cytoplasm to become finely granulated. The later erythro-

blasts exhibit a more condensed nucleus, which stains more deeply, and a larger amount of cytoplasm which becomes more eosinophilic (fig. 38). The further differentiation of erythroblasts into erythrocytes consists in the growth and elongation of the cytoplasmic mass accompanied by elaboration of more hemoglobin. This latter process results in more deeply red-staining cytoplasm. The nucleus becomes smaller, more concentrated, and more deeply staining. The individual chromatin blocks become less distinct. Intravascular erythrocytopoiesis only was observed.

An interesting feature of the erythrocytopoietic process in the lungfish is that pro-erythroblasts and erythroblasts may show nearly as much, and similar, variation in morphology as their hemoblast progenitors. This affords a useful key for determining which kinds of lymphoid cells may act as progenitors of erythrocytes. The evidence indicates that all kinds may function as ancestors, with the probable exception of the smaller lymphocytes with coarse-granule nuclear chromatin (fig. 63). This last type of cell may pass into the circulation as a small lymphocyte; it may transform in the splenic sinuses into a thrombocyte (figs. 90 to 92); or it may degenerate in the spleen without ever reaching the circulation (figs. 78 to 80). But pro-erythroblasts and erythroblasts with similar coarse-granule chromatin nuclei are not in evidence. Thus, we consider the cells of figures 52 to 58, 60, and 61 capable of erythrocytogenesis, but not those of figures 62, 63, and 59.

Thrombocytopoiesis. Thromboblasts and thrombocytes are quite numerous in the region of the splenic pulp. It is obvious that active production of this type of cell takes place in this locus. One variety of mature thrombocyte is approximately spherical or oval (fig. 91). The cytoplasm is relatively scant and may or may not present a faint granulation in eosin-azure sections. In blood smears the outer part of the cytoplasm is delicate and often presents irregular processes; in sections, however, the outer edge is generally smooth and regular. The nucleus is greatly condensed, the chromatin

being in the form of large granules rather closely massed together, so that in many cases some fusion of granules has occurred. Grooving of the nucleus may or may not be visible. It seems typical for a few of the chromatin granules to take the azure stain very deeply, giving a slightly pycnotic appearance.

Another variety of thrombocyte is elongated or spindle-shaped (fig. 92). In such cells the chromatin is drawn out in the direction of the long axis of the cell. There are almost always one or more distinct surface grooves along the nucleus. Acidophilic granulation may be conspicuous at both poles, at one pole, or extending entirely about the nucleus.

The spherical type of thrombocyte predominates. The younger thromboblasts closely resemble the smaller lymphocytes of the periphery of the lymphoid mass. The inference is that the small (or medium-sized) lymphocytes with coarse-granule chromatin function largely as mother cells for thrombocytes. Differentiation takes place only intravascularly, at least as regards the acidophilic granulation of the cytoplasm. The transitional types of small and medium-sized lymphocytes may also become transformed into thrombocytes. However, no cases have been noted which suggest a similar transformation of the large lymphocytes with vesicular nucleus.

Monocytopoiesis. While monocytes are probably formed in the general circulation, as has already been noted, they may also arise in the spleen. They may be derived from lymphoid hemoblasts or from reticular cells. Many become phagocytic, disposing particularly of large numbers of granulocytes and senile erythrocytes. One of these cells is figured (fig. 45) which contains ingested material, but which still presents the young hemoblast nucleus of vesicular type with conspicuous red-staining plasmosome. Similar phagocytic activity is exhibited by monocytes or macrophages in the liver and in the granulocytopoietic capsules of the kidneys (fig. 50) and gonads, and in the wall of the gut.

Granulocytopoiesis. Eosinophils with coarse granules. Eosinophils are formed in great numbers in the wall of the

intestine, in the spiral-valve region. A special granulocytopoietic organ is located at this level (fig. 49). As in the case of the spleen, this organ lies in the submucosa internal to the muscle layers. Anteriorly, this organ reaches the spleen. Posteriorly, it extends practically to the end of the intestine, with some variation in its degree of development. Other granulocytopoietic regions are the capsules of spleen, kidneys, and gonads.

The usual ancestral cell for the coarse-granule eosinophil is the large lymphoid hemoblast with vesicular nucleus (fig. 23). This cell corresponds to the large lymphoid hemoblast with amblychromatic nucleus which has been described in the spleen. In the early stages of development the young eosinophil nucleus is much like that of the hemoblast (fig. 31), and the cytoplasm may contain mixed bluish and reddish granules (figs. 32, 33). Mitoses are frequent (fig. 34). In the later stages the nucleus becomes larger, sometimes indented, lobulated or even double. The granules become larger and the archoplasmic area becomes more distinct (fig. 35). The cytoplasmic ground-substance stains a light grayish blue. The plasmosome may persist quite late in development.

In the spleen also, particularly in the capsular region, young eosinophils may be seen developing. In some cases a hemoblast ancestry is indicated, but in other cases the cells appear to differentiate directly from reticular cells. It is probably true also that in the intestinal wall, kidney, and gonad capsular regions, coarse-granule eosinophils sometimes take origin directly from reticular cells.

Special eosinophils. The special eosinophil is characterized by fine eosinophilic granules and by a nucleus of the polymorphous type (fig. 30). This cell is regarded as the homologue of the neutrophil of higher vertebrates. Special eosinophils may be seen arising in the granulocytopoietic organ of the gut wall. These cells develop from the lymphoid hemoblast with vesicular nucleus. The red granules may or may not be preceded by blue ones (fig. 24). Active multiplication by mitosis of granuloblasts occurs (fig. 25). The nucleus under-

goes some lobulation (fig. 28), often suffering loss of nuclear material in the process. An archoplasmic area sometimes becomes visible in the early stages (fig. 27). Nuclear condensation also is quite characteristic (fig. 28).

In the later stages there is an increase in nuclear lobulation in many but not all of the cells. The proportion of cytoplasm to nucleus increases (fig. 30). In a few cases cells were observed containing coarse granules as well as fine ones (fig. 29).

In the spleen this type of cell is also found in early stages of differentiation. The ancestral cell in this locus is the hemoblast, or it may be a lobulated lymphocyte. It is also probable that the reticular cell may function directly as the mother cell for the special eosinophil in the intestinal wall organ and in the capsules of kidneys and gonads.

Meta-eosinophils. In all of the granulocytopoietic areas there are cells containing granules which exhibit other varieties of staining reactions, such as pink, purple, green, yellow, colorless, and mixed (figs. 39 to 43). Cells of this sort are termed 'meta-eosinophils.' Many of these cells are young and appear in no way degenerate forms. In fact, a number have been seen in amitosis (fig. 39). They appear to be of the same general nature as the coarse-granule eosinophils. The green granulocytes are found only in close proximity to the peritoneum. That some of these cells represent senile or abortive eosinophils seems certain. Faint pink, yellow, and colorless granules occur in cells that are in all probability senile eosinophils. A continuous series of transition stages showing senility in these cells can be traced. Meta-eosinophils are also present in the general circulation.

The occurrence of coarse granulocytes with mixed red and blue granules (fig. 40) is of special interest to us, as these cells are obviously homologous to those previously noted by us in the turtle thymus and spleen (Jordan and Speidel, '28).

Basophils. Young basophils have been seen in the spleen and in the general circulation. They appear to come from the small or medium-sized lymphoid hemoblasts. In a young

basophil the nucleus is spherical or slightly lobulated (fig. 44) and a single row of basophilic granules may be present. An archoplasmic area is not usually visible at this stage. Further development consists in growth of the cytoplasm, formation of more metachromatic granules, and indentation or lobulation of the nucleus. The nucleus becomes hazy and indistinct. While direct origin from the hemoblast may be traced, the general characteristics of basophils suggest degeneration or abortive development.

In the circulation young basophils may sometimes present a bluish granulation as well as the definitive metachromatic granulation. The granules may be fine, coarse, or of intermediate sizes, varying in much the same manner as in eosinophils. Late stages in basophil development or senile stages may be characterized by partial vacuolation, as if a result of liquefaction of the granules. In some regions, as in the intestinal wall, the connective-tissue-cell origin of basophils is conspicuous.

Disposal of granulocyte débris. Correlated with the large number of granulocytes in lungfish blood and certain tissues, there occurs conspicuous granulocyte degeneration. Both eosinophils and special eosinophils fragment in large numbers, the débris being ingested by macrophages (figs. 10, 45). There are progressive changes in the staining reactions of the granules. Various shades of pink, yellow, and brown may characterize the granules, formerly eosinophilic. Some may be quite colorless. These changes may occur inside macrophages or they may occur without macrophage influence. In late stages of change pigment is produced. This may vary from light tan or brown to deep black. Large masses of pigmented material formed from granulocyte débris constitute one of the most prominent features of the spleen. A similar condition is found in the wall of the intestine, in the kidney (fig. 50) and gonad capsules, in the liver, and to a less extent in other loci. Elimination of some of this material appears to take place by way of the intestinal lumen, some also by way of the kidney (figs. 99, 100) and liver cells.

The reticular cells and endothelial cells of the venous sinuses of the spleen seem also to play a part, at least at times, in the disposal of granulocyte débris. Granules which stain a light pink, or which are practically colorless, may be present in the cells of the reticular framework of the spleen and in the endothelial cells of the sinuses (figs. 84 to 86). In view of the extensive granulocyte degeneration, the presumption is that these granules, partly at least, represent former eosinophil granules which have been ingested.

Disposal of erythrocyte débris. Senile erythrocytes are found in the general circulation and in the venous sinuses of spleen, liver, and other organs. They are especially prominent in the spleen pulp. All conceivable stages of degeneration are to be seen of whole erythrocytes, of naked erythrocyte nuclei, and of cytoplasmic fragments. The staining reaction varies from red, green, or yellow to various shades of brown. Some fragments are almost colorless. The degenerating nuclei vary from deep blue, or black, to deep green.

The fragments may be free or they may be in macrophages. Some macrophages seem to specialize on the ingestion of cytoplasmic fragments, some on nuclear material; a few show both kinds; and many show other types of débris, such as granulocyte débris. Pigment formation seems to occur to some extent in the later stages of degeneration. Both reticular cells and the endothelial cells of the venous sinuses contain granules which to some extent may represent erythrocyte débris.

Observations upon other organs

The kidneys. The granulocytopoietic capsule of the kidney contains a variety of granulocytes. Some activity in granulocytopoiesis is apparent, a number of young eosinophils and of young special eosinophils being present.

More conspicuous, however, are the indications of degenerative change. Senile granulocytes are numerous, containing granules of various colors: green, yellow, and pink. Some are colorless. Deeply pigmented material represents a late

stage. Macrophages with ingested material display a tendency to congregate. An occasional mass of lymphoid cells occurs (fig. 50).

Suprarenal cells were not seen in relation to the kidney. Typical chromaffin cells are, however, conspicuous in the heart—a condition which has been described for the lungfish.

The gonads (ovaries). Conditions in the connective-tissue capsule of the ovaries are very similar to those of the kidney, although both granulocytopoiesis and the degenerative processes are less pronounced.

The liver. The liver of the lungfish is not an important organ from the standpoint of blood-cell formation. In the venous sinuses occur some of the younger stages of erythrocytes and thrombocytes, but these are not numerous. An occasional locus of either granulocyte or erythrocyte degeneration may be seen. The liver cells may show pigmented granules, which probably represent the products of erythrocyte degeneration about to be excreted as bile pigment.

The heart. Little of interest was found in the heart from the standpoint of hemocytopoiesis. Unlike the salamander, there occurs no marked number of immature red cells, nor is there any indication of rounding up of reticulo-endothelial cells, as seen by Wituschinski ('28) in the axolotl heart. One interesting feature is the presence of a number of chromaffin cells. These occur in small nests or groups. Cartilage is also quite prominent in the wall of the heart in certain regions.

The stomach. The stomach lining shows migrating granulocytes and macrophages. Near the peritoneum are metaeosinophils with purple granules. Basophils are also numerous.

Bursa entiana. The bursa entiana is located just below the stomach. The internal wall or lining is raised into a number of deeply pigmented folds. The pigmented appearance is caused by numerous cells containing dark pigment granules which appear to be in process of migrating through the lining. Many of these are true pigment cells, but others are macrophages loaded with ingested débris. The lower portion of the

spleen and the upper portion of the granulocytopoietic organ of the intestine may both be seen at this level.

The pancreas. The pancreas is unimportant from the standpoint of hemocytopoiesis. It is in close relation to the spleen and is embedded in the stomach wall in a similar fashion. Its capsule shows some granulocytopoiesis, as does that of the spleen. There may also be seen areas of granulocyte degeneration. Near the peritoneal edge are the characteristic purple granulocytes with coarse granules. The deeply pigmented appearance of the pancreas is caused by numerous pigment cells (chromatophores) scattered throughout the organ.

The lungs. The lungs are rather simple sac-like organs, in general appearance much like those of the salamander. They are not important as regards blood-cell formation or destruction. Granulocytes are conspicuous in the pulmonary capillaries and also in the connective tissue underlying the epithelium.

Vertebral column. Sections through the vertebrae failed to reveal any locus of hemocytopoietic activity. Notochord and cartilage appeared as the only constituents, no trace of marrow being seen.

Muscle. Sections through muscles associated with the vertebral column revealed nothing of interest for this study. Neither in normal nor in estivating animals was anything apparent indicative of blood-cell activity.

The granulocytes of kidney and gonad capsules are in close proximity to perirenal fat, and may be important agents in its transportation. In some preparations the granulocytopoietic area is seen to be continuous with the fat area.

Estivation

Animals subjected to long estivation periods showed marked changes in the blood and hemocytopoietic organs. The best specimen for observation of the blood changes proved to be no. 8, an animal in dry estivation for one year.

As compared with normal animals, the blood contains a large number of degenerating granulocytes (fig. 20). These include both eosinophils and special eosinophils. Early stages in this process are represented by cells in which the granules are somewhat less distinct in outline and the nucleus less sharply defined, with a tendency to stain less deeply. In later stages the cell features are still less distinct. Many cells present a pale-staining watery appearance (fig. 20). The granules in the latest stages become practically colorless, and the nucleus becomes pale blue and almost homogeneous. In a few cases pigment formation accompanies the degenerative process.

Another conspicuous change in the animals subjected to estivation was the absence in the blood of the monocyte. With return of the animals to water, monocytes reappeared in large numbers. This may, however, have been correlated with infection, which attacked animals nos. 21 and 13. There is no trace of mitosis or amitosis of blood cells in the estivating animals. On the other hand, many erythrocytes are to be seen in various stages of senility and degeneration.

In the hemocytopoietic organs changes from the normal condition are also conspicuous. In the spleen and kidney there occurs a great amount of eosinophil destruction. Large masses of degenerating cells with pigment are being handled by macrophages. In the kidney the tubule cells show many deeply pigmented granules passing through them (fig. 99), the inference being that these cells slowly excrete the products of eosinophil degeneration. To a less degree there appears to be similar activity in the liver.

The granulocytes are much more conspicuous in the spleen and intestinal granulocytopoietic organ. In the spleen almost the entire area outside of the central lymphoid mass is packed with eosinophils. The spleen pulp appears to be practically without its normal erythrocytopoietic activity. The cords of lymphoid cells surrounding the spleen pulp in the normal animal had been almost entirely replaced by granulocytes. The intestinal granulocytopoietic organ also appears to be a solid

mass of granulocytes. Although the granulocytes are everywhere conspicuous, there is little or no indication of active formation of new granulocytes. Such activity is at a low ebb.

Mixed-granule eosinophils or meta-eosinophils, i.e., cells containing both basophilic and eosinophilic granules, were noted particularly in the estivating animal no. 8 (fig. 40). Whether or not they are produced as a result of the estivation is not known. It seems to us that these cells constitute merely one more variety of meta-eosinophil.

Animal no. 21, which was replaced in water after a long period of estivation and in addition given an injection of thyroxin, shows active blood and hemocytopoietic organs. In the general circulation occur erythrocytes in all stages of development from hemoblasts (figs. 1 to 4). Senile stages are also quite numerous. The unusual types represented in figures 5 and 6 are present. The intracellular nuclear-degeneration type of erythrocyte was so common in animals nos. 29 and 21 that random sample counts were made. Of 1000 erythrocytes in the general circulation, 235 showed this kind of degeneration in no. 29 and forty-five showed it in no. 21.

Eosinophils and special eosinophils in all stages of degeneration are conspicuous. Monocytes and macrophages are numerous and quite active in the ingestion of débris, especially the remnants of eosinophils and special eosinophils. In animal no. 21 amitoses of hemoblasts (fig. 17) and of monocytes were seen. This latter type of cell is present in all stages of development, the ancestral cell being the hemoblast. The reddish granulation when present is always conspicuous about the archoplasmic area. In some cells the granules are distributed in circular bands near the periphery. Great size variations occur.

The macrophages (figs. 9, 10) are obviously later differentiation forms of monocytes, usually with ingested material in the cytoplasm. These cells ordinarily lack the reddish granulation and archoplasmic area. The cytoplasm appears moderately delicate, presenting a faint granulation, vacuoles, and processes.

In the spleen amitoses of eosinophils and of meta-eosinophils were observed (fig. 39). In certain areas these are quite numerous, distinct cell plates being sometimes visible, as in the cell figured. A large number of eosinophils and meta-eosinophils possess nuclei of great interest. Each nucleus presents several long lobular extensions from a central region, as if under the influence of a strong 'amitotic urge' which is being expressed in a somewhat abnormal manner. Mitoses of eosinophils and special eosinophils are also numerous.

The lymphoid cords of the spleen have an exhausted appearance, as if these areas had been largely drained of their cellular content. This is the appearance also of the splenic pulp and capsular zones.

Stages in the differentiation of erythrocytes, thrombocytes, macrophages, and special eosinophils were seen. Mitoses of hemoblasts occur and lobulation and amitoses of the smaller lymphocytes. In addition to the reticular-cell mode of origin of hemoblasts, animal no. 21 also exhibited their origin from endothelial cells of venous sinuses. These cells undergo typical stages of rounding up and separating from the sinus wall. Sometimes several separate in a group.

The cells of the kidney tubule show vacuoles and granules (fig. 100) that contrast markedly with the condition in animal no. 8, which was not placed in water after the long estivation period. The general active appearance of the kidney of no. 21 suggests that much of the pigmented eosinophil débris that accumulates in estivation is excreted.

Comparison of hemocytopoietic organs of lungfish fry, young lungfish, and adult

In the fry two months old there is as yet no marked definition of central lymphoid core, spleen pulp, and spleen capsule (fig. 47). The spleen shows little or no differentiative activity. Proliferation of hemoblasts dominates the entire organ. Erythrocytes and thrombocytes, therefore, are formed especially in the general circulation and sinuses of the liver.

The kidneys afford the chief locus for granulocytopoiesis, the special eosinophils being formed here in large numbers. Eosinophils and meta-eosinophils with coarse granules are not present here, nor are they to be seen in the general circulation. These types appear only later in development. Basophils, monocytes, and macrophages were not seen. Yolk granules persisting from the egg are in evidence in the cells of liver and notochord, and in association with the posterior part of the alimentary tube.

In the young lungfish two years old the spleen pulp is an active locus for erythrocyte differentiation. Eosinophils with coarse granules are conspicuous, as are also the special eosinophils with fine granules. Monocytes, macrophages, thrombocytes, and basophils are in evidence. Degenerative activity is apparent.

The kidney differs from that of the fry in exhibiting coarse-granule eosinophils as well as cells containing granules of purple, pink, yellow, and other shades. Special eosinophils are also numerous. Degeneration of granulocytes is quite conspicuous, especially in the capsular region, associated with the presence of macrophages and the formation of pigment.

A granulocytopoietic organ is well developed in the intestine, active in the production of eosinophils and special eosinophils.

The largest lungfish in our collection, 60 cm. long and estimated to be six or more years old, shows some advance in hemocytopoietic differentiation over the younger fishes. The spleen is much larger, its regions are more definitely marked out, the central lymphoid core shows lobulation, with some lobules detached from the main mass. The presence of exhausted areas usually at the boundary zone between lymphoid core and spleen pulp is noticeable (fig. 51). The cells, themselves, appear more mature. The special eosinophils show a greater proportion of cytoplasm to nucleus (fig. 30), and the nucleus is more lobulated or polymorphous.

The eosinophils likewise show similar differences, though perhaps not quite so marked. Vacuolation may be exhibited

by both eosinophils and meta-eosinophils. There is more active erythrocyte differentiation in the spleen pulp. Russell-body cells (fig. 46) are more in evidence.

DISCUSSION

The foregoing observations on the hemocytopoietic organs and blood of the lungfish reveal a number of interesting features which are worthy of special discussion. The lungfish is particularly notable for the transitional position of the spleen from the phylogenetic viewpoint; the general resemblance of its blood to that of urodeles; the variety of lymphoid cell types in the spleen of the adult; the number and variety of granulocytes, including meta-eosinophils; the decided effects on the blood and hemocytopoietic organs of prolonged estivation and recovery.

Hemocytopoietic loci and the phylogeny of the spleen

In vertebrate evolution the spleen is represented in cyclostomes by scattered cords of hemocytopoietic tissue in the wall of the alimentary tube. Each cord has a central venous sinus region where erythrocytopoiesis takes place and a peripheral region where granulocytopoiesis occurs. This is the condition in the hagfish Myxine (Jordan and Speidel, '30). In another cyclostome, the lamprey, the hemocytopoietic tissue is somewhat more localized, and is aggregated into a typhlosole-like mass which projects into the dorsal wall of the intestine. The centrally located arteries are ensheathed by lymphoid tissues. Surrounding this is a region of pulp cords and sinuses, which may completely encircle the intestine. Granulocytes are also produced in this region.

In the lungfish a further advance is to be noted. The spleen is here a very definite organ, but it is nevertheless embedded in the wall of the stomach, being covered externally by the muscle of the gastro-intestinal wall. The rather sharp distinction between central lymphoid mass, intermediate splenic pulp, and peripheral cords has already been pointed out. While the spleen is to some extent a granulocytopoietic organ,

this function has been largely assumed by other regions, such as the granulocytopoietic organ of the intestine and the capsule of the kidneys and gonads.

A still further advance in spleen evolution is represented by the conditions in the other vertebrates in which the spleen becomes entirely separate from the alimentary tube wall. Thus the condition of the lungfish, with the spleen as a definite compact organ, but still embedded in the stomach wall, may be considered as a transitional stage between the scattered hemocytopoietic tissue of the cyclostomes in the gut wall and the compact spleen of other vertebrates entirely separated from the alimentary tube.

The lungfish with its primitive salamander-like lungs foreshadows the amphibian types. It is interesting to note that the blood of the lungfish bears marked resemblances to that of the salamander (compare Jordan and Speidel, '30). Although blood cells of most fishes are of small size, those of the lungfish are enormous, being of a size comparable to those of urodeles. Other resemblances that may be mentioned are the similarities in the erythrocytopoietic process, the various immature cells being much alike at similar stages; the occurrence in the general circulation of immature cells of the erythrocyte series; the similar degenerative types of erythrocytes; the similarities of the monocytes, basophils, eosinophils, and thrombocytes.

Erythrocyte amitosis in the lungfish also seems entirely similar to that described for Necturus (Charipper and Dawson, '28). Among the differences in the two forms may be mentioned the great variety of meta-eosinophils in the lungfish and the difference in locus of granulocytopoiesis; in the lungfish the alimentary tube wall, kidney, and gonad capsules, in the salamander the capsule of the liver.

The lymphocyte types and their hemocytopoietic capacity

The various types of lymphoid cells have already been described. These are of such variety and of such size in the lungfish that a study of their hemocytopoietic capacity is

much more readily made than in most animals. Special attention has therefore been paid to the early pro-erythroblasts and erythroblasts to obtain information as to their probable origin. It seems entirely clear that the usual erythrocyte mother cell is the large or medium-sized hemoblast with typical vesicular sieve-like nucleus containing one or more acidophilic plasmosomes. Both pro-erythroblasts and erythroblasts with nuclei of this type occur in large numbers. The plasmosomes often may be seen in late erythroblast stages. The nucleus of pro-erythroblast and erythroblast may sometimes be slightly lobulated, as may that of the hemoblast mother cell.

Lymphocytes which correspond to the small lymphocyte of higher vertebrates do not appear to give rise to erythrocytes. These cells have the pachychromatic type of nucleus with coarse chromatin blocks, cart-wheel arrangement, and very little cytoplasm. Pro-erythroblasts and erythroblasts never present nuclei of this sort. The fate of some of these cells is to undergo progressive degeneration (figs. 76 to 80). Others circulate in the blood stream as small lymphocytes. But a large number migrate into the splenic sinuses and there undergo transformation into thrombocytes. Transition stages are numerous between the vesicular-nucleus type of hemoblast and the coarse-granule nucleus types. The real problem is to what extent these transition stages may retain their original erythrocytopoietic capacity. Judging from the character of many of the earliest pro-erythroblast nuclei, it seems probable that these transitional forms may also give rise to erythrocytes.

The evidence is somewhat similar for the origin of granulocytes and monocytes. In each case the usual ancestral cell is the vesicular-nucleus type of hemoblast. However, the transitional lymphoid cell types can by no means be eliminated as potential granulocytes, monocytes, and thrombocytes.

An interesting feature of the lymphoid mass of the lungfish spleen is that it presents small hemoblasts with vesicular nucleus (i.e., 'small-sized large lymphocytes') and large lym-

phoid cells of the small lymphocyte type (i.e., 'large-sized small lymphocytes'). Small-sized hemoblasts like those of figures 60 and 61 were noted by Danchakoff ('16) in her experimental work on chick allantoic grafts. Such small hemoblasts were interpreted by her as resulting from intense mitotic activity on the part of larger hemoblasts.

In a recent study of the spleen in a number of fishes, including elasmobranchs, teleosts, and a single specimen of lungfish, Calamoichthys,[2] Yoffey ('29) concludes that the small round lymphoid cell is the progenitor of erythrocytes. An examination of his figures showing erythrocytopoiesis, however, does not seem to bear out this idea. Unfortunately, Yoffey makes no mention of thrombocytes in the spleen—cells which are sometimes confused with small lymphoid cells. His figure 4, plate 3, represents a cell, which he interprets as a transition form between a small lymphoid cell and an erythroblast. This cell to us, however, appears to be a fairly typical young thrombocyte. Characteristic features are the acidophilic cytoplasm faintly granulated, and the 'grooved' nucleus with chromatin drawn out somewhat into threads. We have seen many thrombocytes of this type in the spleen, both in lungfish (fig. 91) and in salamander. Furthermore, the cell of his figure 5, an early erythroblast, presents a nucleus which to us indicates a probable derivation from the large lymphoid cell with vesicular type of nucleus. Yoffey's series of figures begins with a cell containing coarse chromatin granules (fig. 1), which develops into a cell with finer granules and more vesicular nucleus (fig. 5), which then leads to a coarse-granule type (figs. 6, 7). The early part of this series seems questionable.

Seasonal and regional activity of the spleen

In animals which undergo periods of hibernation and reawakening, such as the frog, salamander, turtle, and horned toad, we have already pointed out that there occur cyclical or

[2] While Calamoichthys is sometimes classified with the lungfishes, it is perhaps more usually classified with the ganoid fishes.

seasonal hemocytopoietic changes. This holds true also for the lungfish, though in this case the torpid state is induced by lack of water rather than by cold.

Not only is there evidence of seasonal variations, but there is also evidence of regional activity in the spleen, followed sometimes by exhaustion and regeneration. In animal no. 2 the spleen is very active. Many small areas are visible in the spleen pulp zone, or in the boundary zone between central lymphoid mass and spleen pulp, from which practically all cells have been drawn with the exception of the reticulo-endothelial framework. Two of these areas may be seen in the low-power photograph (fig. 51), though the condition is much more striking with higher magnification.

In animal no. 21 the entire spleen appears relatively exhausted, as if very recently drained of its cellular content. This is somewhat like the splenic exhaustion induced in frog tadpoles by thyroid treatment, and in frogs by experimental anemia (Jordan and Speidel, '23 and '24). In some areas there is evidence of regenerative process, such as we have described for the horned toad (Jordan and Speidel, '29).

Significance of erythrocyte amitosis

The amitotic activity exhibited by the erythrocytes of lungfish no. 1 is so striking that it must be of significance. This animal, five days in water after two months in a small can of earth, is in an early stage of recovery from the torpid condition. Increased metabolic rate and respiratory rate place an added burden on the erythrocytes as oxygen-transporting agents. Multiplication of erythrocytes, therefore, would seem to be a natural physiological adjustment accompanying the change from torpid to active state. Not a single example of erythrocyte division by mitosis, however, was seen in the blood of this animal; only the amitotic type was in evidence. Contrasting sharply with this is the condition in normal well-fed lungfishes, nos. 33 and 34, in which erythrocyte mitoses were common and amitoses not to be seen.

Observations on salamanders are of interest in this connection. Mitosis of erythrocytes is the rule, both in normal animals and in splenectomized animals. However, Dawson ('28) has observed that erythrocyte amitosis occurs in Necturus blood plasma which has been allowed to stand for several days. Cases have also been found in normal Necturus blood.

These observations strongly suggest that amitosis represents a rapid response to a sudden change in environmental conditions. It is of the nature of a sudden emergency reaction. Its duration is short-lived. Mitosis represents the response to more gradual change; it represents the method of normal replenishment, and of replenishment over a long period of time, as in year-old splenectomized salamanders. In general, also, amitosis appears in relatively mature cells; mitosis, in less mature cells—a fact which fits in with the idea just stated. In sudden emergencies fully differentiated cells are in demand. In more gradual crises the younger less differentiated cells, which are easily susceptible to mitosis, are adequate to multiply, then differentiate in time to care for the deficiency created by the changed conditions.

Another interesting feature of animal no. 1, that should be mentioned in this connection, is that mitoses of young leucocytes are very common in the granulocytopoietic organ of the intestine. This contrasts sharply with the occurrence in this animal of erythrocyte amitosis only. The deficiency of leucocytes created by the return of the animal to water is not so critical as the deficiency of erythrocytes. Thus, the leucocyte deficiency is cared for by the normal granulocytopoietic regions with somewhat heightened mitotic activity.

In animal no. 21 a more critical leucocyte condition is presented than in animal no. 1. The long estivation period of 427 days, the thyroxin injection, and the infection after return to water are all contributing factors. Monocyte and granulocyte amitoses are here regarded as emergency reactions elicited by these factors. Granulocyte mitoses are also exhibited by this animal.

The granulocytes; senility and relation to pigment formation

In many ways the granulocytes in the lungfish are of exceptional interest. They are by all odds the most plentiful and of the greatest variety we have seen in any vertebrate. They arise from connective-tissue cells in many localities, and may also arise from hemoblasts. Furthermore, they degenerate in great numbers, accompanied by pigment formation and ingestion by macrophages. They also appear to play an important rôle during the estivation periods.

Senility in an eosinophil of the coarse-granule type may be exhibited in a variety of ways. In some cells the granules become progressively less eosinophilic, staining various shades of light red or pink, or orange or yellow, and finally become almost colorless. The nucleus at the same time takes the stain less and less. Or the granules may be replaced by (possibly transformed into) pigment granules. A few cells containing both eosinophil granules and pigment granules have been observed both in the lungfish and in the salamander. In general, however, pigment granules are formed after phagocytosis by macrophages. Or, the granules may become atypically basophilic. A number of eosinophils have been seen in the granulocytopoietic organs which contain granules showing various degrees of basophily. This color reaction does not appear to be quite a normal basophilic color. The occasional presence also in these cells of colorless granules suggests that they are about to degenerate. Similar cells have been noted in the thymus of the turtle (Jordan and Speidel, '28) and also in the spleen.

Still another way in which granulocytes may become senile is by method of cytoplasmic segmentation or nuclear extrusion. Examples of large cytoplasmic fragments have been noted in the circulation; also a few nuclei somewhat degenerate with small cytoplasmic fragments still attached which show their eosinophil-granule nature. Free red granules from eosinophils are commonly to be seen in the spleen and other regions.

Senility of the special eosinophils is also easily followed. There is here, too, a tendency for the granules to become colorless in old worn-out cells. Pigment formation was not seen in these except after ingestion by macrophages. Whether the plentiful occurrence of degenerating granulocytes in the estivating lungfish means that these cells have a specific relation to this process is not clear.

The ingestion of degenerating eosinophils by monocytes or macrophages is very conspicuous in spleen, kidney, intestine, and also may occur in the capsule of the gonad, in the liver, lungs, gills, and elsewhere. In the fish subjected to a year of estivation it is even more evident. The nuclear and cytoplasmic débris is broken down in the macrophage and transformed into pigment, at first a golden brown or yellow, then later a deep brown or black. The nuclear material may be immediately transformed into the deeper shades of pigment. In the kidney and liver, excretion of pigment, presumably partly eosinophil pigment, takes place. In the spleen there is also ingestion of free eosinophil granules by the cells of the reticulo-endothelial system.

Granulocytes and fat metabolism

That the granulocytes in the lungfish may be of special importance in relation to fat metabolism seems probable. Unfortunately, living specimens were not available for tests on this question. However, the Nile-blue-sulphate treatment shows in salamanders and in frog tadpoles that the eosinophils are loaded with neutral fat or fatty acid in the form of granules. These granules appear to be the same as the eosinophilic granules. Ready intracellular transportation of fat is thus assured.

The various colors of eosinophil and meta-eosinophil granules in the lungfish, as brought out by the eosin-azure stain, may depend largely on the state of the fatty material; i.e., whether it is in the form of neutral fat, or saturated or unsaturated fatty acid. An excellent comparison of the staining reactions of yolk granules is afforded by our sections of lung-

fish fry. In a two-months-old specimen there is a great deal of yolk material present. This is particularly prominent in sections near the caudal part of the body, but may also be noted in liver and notochord. The yolk granules stain various shades of pink, red, green, purple, yellow, and some may even be colorless. These shades of staining reaction are somewhat comparable to those of the granules of the granulocytes, especially the meta-eosinophils. It is probable that the granules of these granulocytes contain chemical compounds much like those of the yolk granules.

We are thus inclined to interpret the rich variety of granulocytes as being related to the estivation habit, the granules being partly composed of fat or fatty materials and affording an intracellular mechanism for fat transportation.

An interesting point concerning granulocytes is that neither the eosinophil nor the meta-eosinophil is present in the two-months-old specimen. All eosinophils appear to be of the special eosinophil variety containing only the fine granules. Apparently the time factor is of importance. That is, the large granules are formed by the accumulative process from smaller ones. Coalescence has often been seen. In the fry not enough time has elapsed for the coarse-granule formation. In the older specimens subjected to estivation coarse granulocytes of all varieties are numerous.

Another hypothesis suggests itself in this connection. In the fry there is still a plentiful amount of granular yolk material from the egg. Correlated with its disappearance is the appearance of the eosinophils and meta-eosinophils. These cells in the adult may play a rôle similar to that played by the yolk granules in the fry; i.e., to furnish a supply of partly formed material (fats, fatty acids, and other compounds) available for the metabolic needs of the body. In other vertebrates, likewise, the eosinophils make a relatively late appearance; e.g., in the chick Sugiyama ('26) states that they appear only in the last few days of incubation.

Monocytes and macrophages

The lungfishes subjected to dry estivation followed by return to water have proved to be excellent for the study of monocytes. All stages in development of these cells are found in the circulation. The two kinds of monocytes with respect to presence or absence of reddish granulation of the cytoplasm occur. Also, numerous definite transition stages indicate the further development of the monocyte into a full-fledged macrophage. Clark and Clark ('30) have recently reported such transition by direct observation on living frog tadpoles.

The significance of the large number of monocytes and macrophages in the circulation of our estivation specimens is not entirely clear. It may be merely a reaction to superficial infection which was obvious in some animals. Or it may represent a reaction elicited by the large number of degenerating granulocytes, both of the coarse eosinophilic and special varieties. This latter idea is probably correct, as no infection was observed in some animals. However, since our estivating specimens are few in number, any conclusion is necessarily uncertain.

Thrombocytes

The observation that the small lymphocyte in the lungfish is the usual mother cell for the thrombocyte contrasts with the condition in the salamander, in which the usual mother cell is the large hemoblast. This condition is strikingly reflected in the splenic lymphoid cell content in the two animals: the small lymphocyte predominating in the lungfish, the large lymphocyte in the salamander. Fundamentally, this merely means that in the lungfish, nuclear differentiation (i.e., development of coarse-granule chromatin and lobulated or grooved nucleus) may precede the appearance of the acidophilic granules in the cytoplasm. In the salamander the acidophilic granulation may appear before the nucleus differentiates or changes from the primitive hemoblast type. Another point of interest is that the small spherical type of

thrombocyte predominates in the lungfish, whereas the large-sized elongated type is quite numerous in the salamander. This difference may be correlated with the type of progenitor in each case.

Probably not enough emphasis has been placed on the two types of thrombocytes in vertebrates. As has been indicated, the lungfish presents a small spherical or oval type of cell and an elongated or spindle type. Similar types have been observed in the frog, in salamander and several other urodeles, and in cyclostomes. We have not been certain as to the relative age of the two types. In general, the small spherical condensed type seems a bit more highly differentiated. But it does not necessarily pass through the elongated stage in its development. The clumping tendency is exhibited to a greater degree by the small condensed type. This type also shows most clearly the delicate outer zone of cytoplasm which fuses readily in clumping.

The condition in the hagfish is of great interest in this connection. We have pointed out that in this primitive form a rather unique spindle-shaped cell is present in large numbers, besides a typical thrombocyte of the spherical condensed kind (Jordan and Speidel, '30). The former type displays relatively little clumping tendency, and it is quite doubtful whether it should be classified as a thrombocyte. However, it must greatly aid the clotting process by its mere structure with elongated processes. This type greatly outnumbers the clumping type of spherical thrombocyte. In the lungfish and salamander the two kinds are equally conspicuous. In frogs the clumping type is more numerous, and in higher vertebrates it is still more so. Thrombocyte evolution thus appears to present a progressive numerical decrease in the elongate type of 'morphological thrombocyte' which aids clotting by its structure; and progressive increase in the spherical condensed type of 'physiological thrombocyte,' which aids clotting by the release of substances which take part in fibrin formation.

Double origin of pigment cells

Observations on the lungfish emphasize once more the possible double origin of pigment cells. That many of the pigment cells are formed directly from their mesenchymal ancestors is clear, and some derive their pigment directly from the egg. The degeneration of eosinophils gives another possible origin of pigment cells. In fact, some cells have been seen which contain eosinophil granules and pigment granules with no particular evidence of degenerative change.

Much of the pigment in spleen, kidney capsule, gonad capsule, alimentary tube, and other loci is the result of granulocyte degeneration. Macrophages may or may not assist in the production of this pigment.

This process of eosinophil degeneration into pigment which is so pronounced in lungfish is now recognized as also being present in many other forms we have studied, such as frog, turtle, and salamander. It is probable that the pigmented débris of this sort so prominent in hemocytopoietic areas has often been wrongly interpreted as products of erythrocyte degeneration.

SUMMARY

The blood cells of the African lungfish, Protopterus ethiopicus, are very large and in many respects resemble those of urodeles. Leucocytes are especially plentiful and rich in variety, including eosinophils, special eosinophils, 'meta-eosinophils,' monocytes, thrombocytes, lymphocytes, and basophils.

The chief hemocytopoietic organs are the spleen, kidneys, and intestine. Erythrocytes are formed in the splenic pulp, the usual mother cell being the lymphoid hemoblast with vesicular, sieve-like nucleus. Eosinophilic and special eosinophilic granulocytes are formed in the granulocytopoietic organ of the spiral-valve region of the intestine, to a less extent in other loci, such as the capsule of kidneys, gonads, and spleen. Meta-eosinophils with granules showing atypical staining reactions are also formed in the granulocyto-

poietic areas. These are thought to be related to fat metabolism, which becomes of special importance in the lungfish in relation to estivation.

Lymphocytes of all types arise in the spleen. Intravascularly in the splenic sinuses, thrombocytes are differentiated from the smaller lymphocytes. Monocytes are differentiated in the spleen and general circulation. Basophils are formed in the spleen and intestinal wall. 'Russell-body' cells are conspicuous in the spleen.

Macrophages and cells of the reticulo-endothelial system are especially active in the ingestion and disposal of senile granulocytes, particularly in the spleen, kidneys, and intestine. Elimination of this débris largely in the form of pigment appears to be by way of the kidneys, intestine, and liver.

In fishes subjected to long periods of dry estivation, the granulocytes become more prominent throughout the tissues and in the circulation. Many undergo degeneration. Erythrocytopoiesis practically ceases. Senile erythrocytes often present the appearance of intracellular nuclear degeneration.

Fishes replaced in water after estivation may exhibit general recovery of the hemocytopoietic tissues. Erythrocyte amitoses may become very numerous. Amitosis has also been observed in the case of thrombocytes, granulocytes, monocytes, and lymphoid hemoblasts.

The spleen of the lungfish, embedded in the wall of the stomach internal to the muscular layers, represents an intermediate phylogenetic stage between the disperse intra-enteral type of the hagfish and the aggregate extra-enteral type of other vertebrates.

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PLATE 1[1]

EXPLANATION OF FIGURES

Figures 1 to 22 represent cells from blood smears (Wright's stain); 23 to 46 represent cells from sections (azure II-eosin stain). The cells in figures 1 to 7, 9 to 11, 17, 20, 39, and 45 are from lungfish no. 21; 8, 12 to 16, 18, 19, 21 to 29, 31 to 35, 40 to 42, and 44 are from lungfish no. 1; 30, 36 to 38, and 46 are from lungfish no. 2; 43 is from lungfish no. 8. While all cells have been drawn to the same scale, some differences in size are accounted for by differences in the degree of spreading of cells in the smear preparations. × 750.

1 Pro-erythroblast. An early stage in the erythrocyte series. It may be distinguished from the hemoblast by the characteristic deep blue nucleus with granulated appearance. The cytoplasm is usually non-homogeneous, often faintly granular. The nucleus of the lymphoid hemoblast is more purple or light blue (compare figs. 7, 11, 17).

2 Transition stage between pro-erythroblast and erythroblast. Condensation of the nucleus is apparent.

3 Erythroblast with obvious hemoglobin production. Further contraction of nucleus.

4 Mature erythrocyte.

5 Erythrocyte showing 'intracellular nuclear degeneration.' The nucleus presents a typical inflated appearance.

6 Erythrocyte showing nuclear fragmentation. The granules represent chromosome remains, degeneration having taken place during mitosis.

7 Lymphoid hemoblast. The nucleus presents a purplish tinge.

8 Monocyte with typical archoplasmic area near nuclear indentation and reddish granulation throughout the cytoplasm, but most conspicuous about the archoplasmic area.

9 Monocyte (or macrophage) showing no reddish granulation or archoplasmic area. Cytoplasmic vacuoles and processes are conspicuous. This is a more highly differentiated cell than that of figure 8.

10 Macrophage with ingested material, some of it representing eosinophil débris.

11 Lymphoid hemoblast. The nucleus stains a light blue.

12 Thromboblast with mixed reddish and bluish granules in the cytoplasm. 'Grooving' of the nucleus is slightly indicated in two places.

13 Elongated thromboblast.

14 Mature thrombocytes, showing the characteristic clumping reaction with fusion of the delicate pale blue peripheral cytoplasm. The reddish granulation is apparent in one cell at both poles, in another at only one pole, and in the third it is absent entirely.

15 Thromboblast in amitosis. The lower half of the nucleus shows characteristic surface grooves, and the cytoplasm exhibits the thromboblast type of mixed blue and red granulation.

[1] Many of the figures of plates 1 and 4 were drawn by Alice Clark Mullen.

16 Erythrocyte in amitosis. The cell-plate region indicates definite separation between the daughter cells.

17 Lymphoid hemoblast in amitosis. This cell may also be interpreted as an early transitional stage from a lymphoid hemoblast toward a young monocyte.

18 Eosinophil.

19 Special eosinophil.

20 Senile special eosinophil.

21 Basophil with coarse granules.

22 Small lymphoid cell with acidophilic inclusions.

23 Hemoblast with vesicular sieve-like type of nucleus, containing acidophilic plasmosome (taken from granulocytopoietic organ of the intestine).

24 Young special eosinophil (granulocytopoietic organ of intestine).

25 Young special eosinophil in mitosis (granulocytopoietic organ of intestine).

26 to 28 Young special eosinophils from spleen. Condensation of nucleus, with sometimes some extrusion of nuclear material, occurs.

29 Special eosinophil containing some coarse as well as fine granules. The nucleus is of the ring type.

30 Mature special eosinophil from large lungfish, showing small nucleocytoplasmic ratio. An archoplasmic area is visible.

31 Young eosinophil with typical vesicular nucleus, showing acidophilic plasmosome (granulocytopoietic organ of intestine).

32 and 33 Young eosinophils containing mixed blue and red granules (splenic capsule).

34 Young eosinophil in mitosis.

35 Young eosinophil with full-sized granules. An archoplasmic area is visible.

36 Erythroblast (splenic sinus).

37 Erythroblast in mitosis (splenic sinus).

38 Later erythroblast with acidophilic plasmosome still persisting.

39 Meta-eosinophil in amitosis (spleen).

40 Meta-eosinophil with mixed red and blue granules (granulocytopoietic organ of estivating lungfish).

41 Meta-eosinophil with coarse purple granules. Nuclear extrusion is visible (spleen capsule).

42 Meta-eosinophil with coarse green granules (spleen capsule).

43 Meta-eosinophil with fine green granules (spleen capsule).

44 Young basophil (spleen).

45 Macrophage with ingested materials. The nucleus is of the hemoblast type with acidophilic plasmosome and vesicular appearance.

46 'Russell-body' cell (spleen).

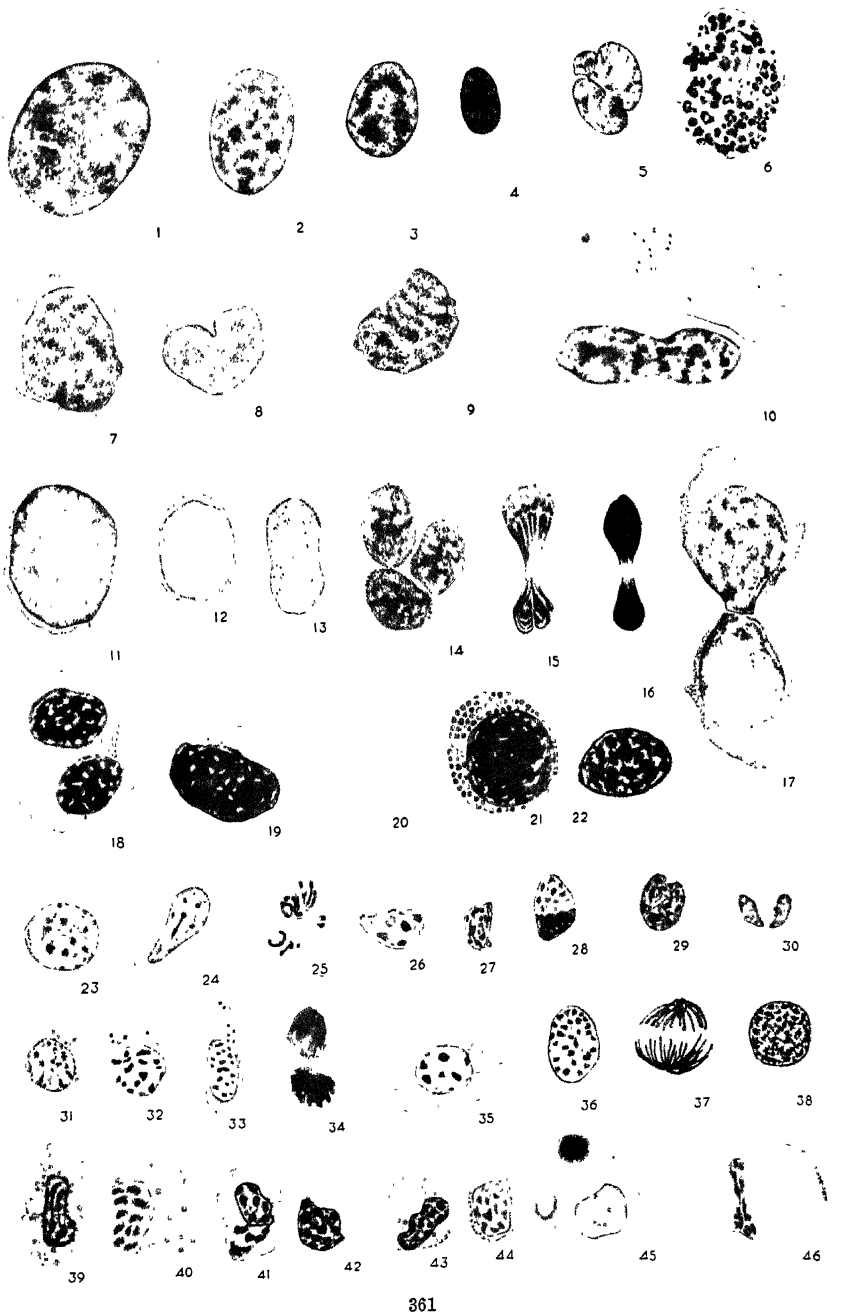
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PLATE 2

EXPLANATION OF FIGURES

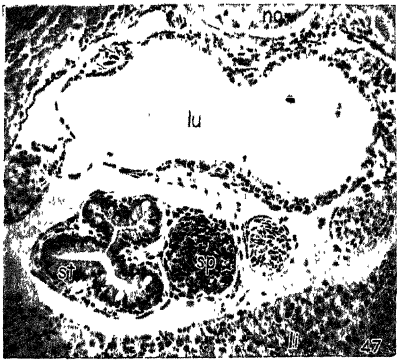
47 Photomicrograph of a transverse section, showing the developing spleen (*sp*) in the lungfish fry. To the left is the stomach (*st*); below (ventral) is the liver (*li*); above (dorsal) are the lungs (*lu*) and notochord (*no*).

48 Photomicrograph of a transverse section, showing the stomach (*st*), spleen (*sp*), and pancreas (*pa*) in a young lungfish (no. 1). The spleen shows well-marked central lymphoid mass becoming lobulated, surrounded by splenic pulp (*pu*), and a thin outer capsule (*ca*).

49 Photomicrograph of a transverse section, showing the granulocytopoietic organ (*gr*) in the intestinal wall of a young lungfish (no. 1). Above are lumen (*lm*), mucosa (*mc*), submucosa, and muscle, below are muscle (*mu*) and peritoneum (*p*). The lobules of granulocytopoietic organ constitute the largest part of the intestinal wall.

50 Photomicrograph of a sagittal section through the kidney of an estivating lungfish (no. 8). Above are the kidney tubules (*tu*); below is the granulocytopoietic capsule (*ca*), which in this case shows also much pigmentary degeneration of granulocytes and macrophages. In the upper right-hand corner a glomerulus is visible; to the left of the center is a lymphoid nodule; below is the edge (*e*) of the kidney. One of the tubules of this kidney is shown under higher magnification in figure 99.

51 Photomicrograph of a small part of an adult spleen (fish no. 2), showing lymphoid mass (*ly*) above, spleen pulp (*pu*) in center, and peripheral cords (*pc*) and spleen capsule (*ca*) below. Several exhausted areas (*ex*) are visible in the boundary zone between lymphoid mass and spleen pulp.



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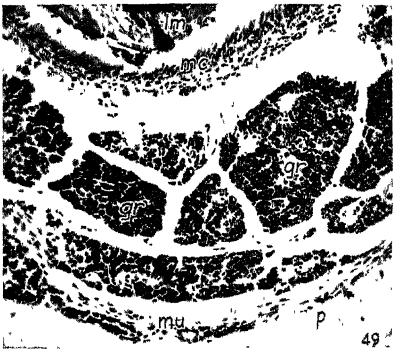

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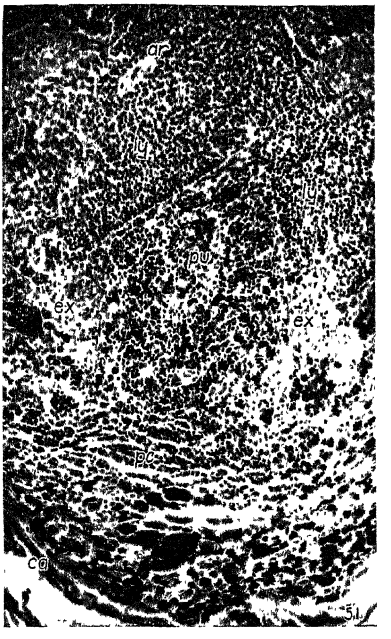

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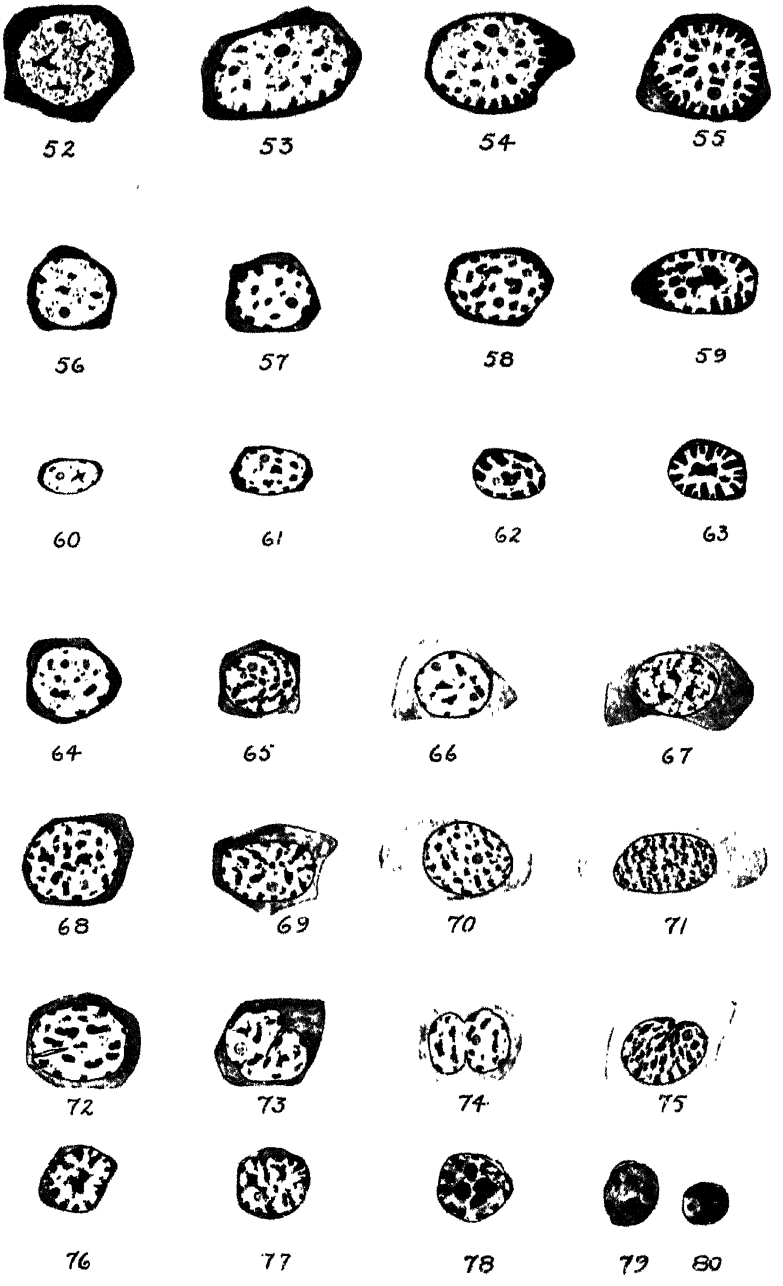
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PLATE 3

EXPLANATION OF FIGURES

All of the cells in this plate were taken from the spleen of animal no. 2, and drawn to the same magnification, × 1000. Figures 52 to 63 show the variations in types of lymphoid cells. Figures 64 to 75 are given to show how erythroblasts may vary in somewhat the same manner. Figures 76 to 80 indicate a common fate of the small lymphocytes with the coarse-granule type of nucleus.

52 Large hemoblast with vesicular sieve-like nucleus (amblychromatic nucleus). Acidophilic plasmosome.

53 and 54 Transitional forms between cells of figures 52 and 55.

55 Large hemoblast with small lymphocyte-like type of nucleus, the chromatin granules being large and numerous (pachychromatic nucleus) and showing radial arrangement. Acidophilic plasmosome.

56 Medium-sized hemoblast with vesicular (amblychromatic) nucleus. Acidophilic plasmosome.

57 and 58 Transitional medium-sized hemoblasts between the cells of figures 56 and 59. Acidophilic plasmosome.

59 Medium-sized hemoblast with small lymphocyte type of nucleus (pachychromatic nucleus). Acidophilic plasmosome.

60 Small-sized hemoblast with vesicular type of nucleus (amblychromatic nucleus). Acidophilic plasmosome.

61 and 62 Transitional forms between cells of figures 60 and 63.

63 Small-sized lymphocyte (pachychromatic nucleus) with coarse chromatin granules radially arranged ('cart-wheel' type).

64 Vesicular lymphoid hemoblast.

65 Vesicular hemoblast in early prophase of mitosis.

66 Pro-erythroblast showing typical vesicular-type nucleus.

67 Erythroblast showing typical vesicular-type nucleus. This cell should be compared with the erythroblasts of figures 71 and 75.

68 and 69 Hemoblasts of transitional type.

70 Early erythroblast with nucleus somewhat similar to those of figures 68 and 69.

71 Later erythroblast.

72 Hemoblast with grooved or lobulated nucleus.

73 Pro-erythroblast with grooved or lobulated nucleus.

74 Young erythroblast with grooved or lobulated nucleus.

75 Erythroblast with grooved nucleus. The acidophilic plasmosome is still present.

76 Typical small lymphocyte with coarse chromatin granules radially arranged.

77 Lobulated small lymphocyte. A small acidophilic plasmosome is present.

78 Small lymphocyte showing early degeneration of the nucleus.

79 Degenerating small lymphocyte.

80 Late stage of degeneration of small lymphocyte.

PLATE 4

EXPLANATION OF FIGURES

Figures 81 to 92 represent cells from the spleen of lungfish no. 2; 93 to 98 are from blood smears of the general circulation of lungfish no. 1; 99 and 100 represent kidney tubules from animals nos. 8 and 21. × 1000.

81 Reticular cell from the reticular syncytium of the spleen. The vesicular sieve-like nucleus with plasmosome bears a marked resemblance to that of the hemoblast.

82 Reticular cell with fibers showing in the cytoplasm.

83 Hemoblast.

84 Reticular cell containing acidophilic granules in the cytoplasm.

85 Reticulo-endothelial cell containing acidophilic granules in the cytoplasm.

86 Endothelial cell lining a vein sinus. Most of these cells show the acidophilic granules in the cytoplasm.

87 to 89 Stages suggesting amitosis of small lymphocytes in the spleen.

90 Lymphocyte with nucleus of the pachychromatic type, for comparison with the thrombocytes of figures 91 and 92.

91 Thrombocyte of the spherical type. This type of cell is easily confused with lymphocytes in sections. The cytoplasm, however, stains less deeply and in well-differentiated material faint acidophilic granulation may be present. Grooving of the nucleus may often be seen.

92 Thrombocyte of the elongated spindle type. This cell, if seen in transverse section, might be mistaken for a small lymphocyte.

93 Early stage of amitosis in an erythrocyte. The nucleus shows elongation and a transverse groove near the middle. This middle area appears less dense. The cytoplasm shows no elongation or constriction.

94 Early stage of erythrocyte amitosis, slightly later than that of figure 93. The nuclear halves show definite separation, leaving a central light transverse area. The cytoplasm shows constriction near the middle.

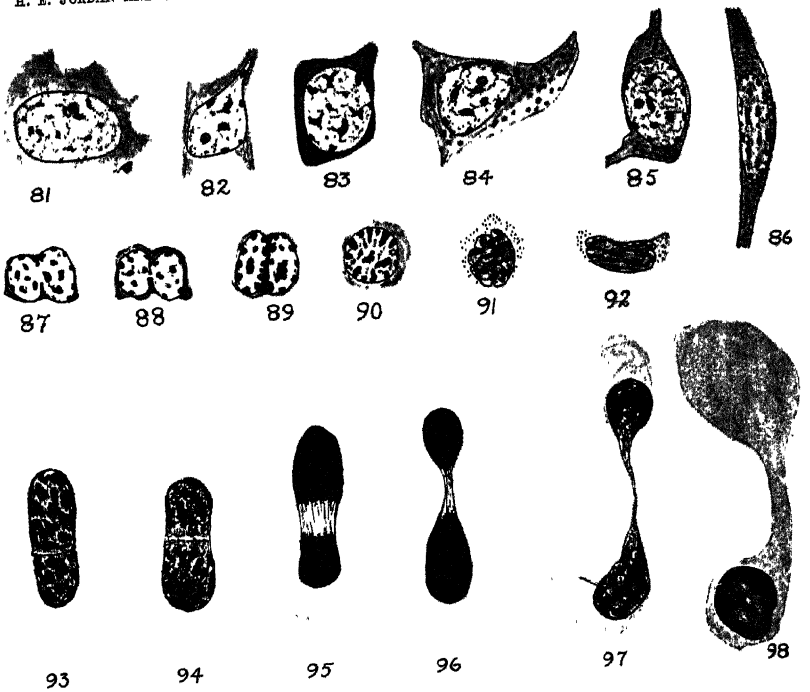
95 Erythrocyte amitosis; later stage than that of figure 94.

96 and 97 Successively later stages in erythrocyte amitosis. Compare also with figure 16.

98 Erythrocyte showing the 'amitotic urge,' the nucleus not, however, being involved. This results in cytoplasmic segmentation.

99 Kidney tubule from animal no. 8 after dry estivation for one year. Three large masses of pigment granules are shown just outside the tubule. This is largely débris of granulocytes. Deeply pigmented granules are also in process of excretion by the kidney tubule cells. Compare with figure 100.

100 Kidney tubule from animal no. 21, showing conditions after 427 days of dry estivation followed by replacement in water for ten days. The kidney tubule cells are vacuolated and in places show light tan pigment granules. The whole appearance of this kidney suggests active excretion. Most of the deeply pigmented débris, such as shown in figure 99, has apparently been eliminated.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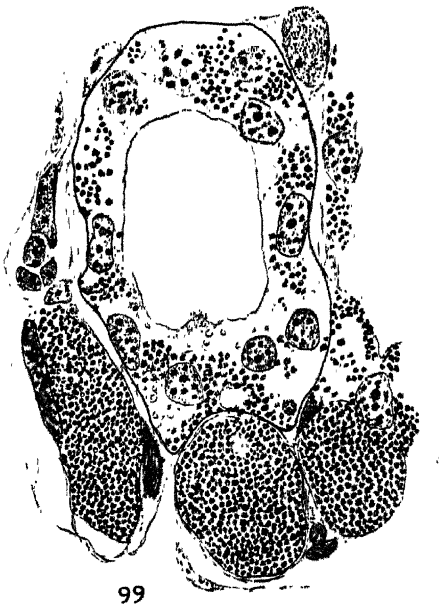
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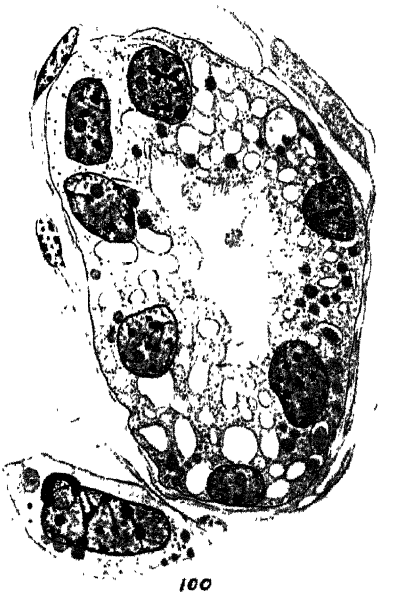
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THE EVOLUTIONAL SIGNIFICANCE OF THE CHROMOSOMES OF APHIDIDAE

ORIHAY SHINJI[1]

ONE TEXT FIGURE AND FIVE HELIOTYPE PLATES (ONE HUNDRED EIGHTY-NINE FIGURES)

AUTHOR'S ABSTRACT

An investigation into the number of chromosomes in thirty-seven species of Aphididae belonging to twenty-seven genera has shown that there is but one sex chromosome in the male cells of all but one species of Aphididae. That exceptional species is Euceraphis betulae Koch.

The number of chromosomes and body characters are so closely correlated that we can safely judge the evolutional scale of any aphid by its number of chromosomes.

In Aphididae the least number of chromosomes seems to be the most primitive.

The number of chromosomes varies with the genera. The highest number is $18_{II} + X = 37$ chromosomes, the lowest being $2_{II} + X = 5$ (diploid in male).

The increase in the number of chromosomes seems to have been brought about by transverse divisions of the primitive chromosomes.

The genus Tuberolachnus with $3_{II} + X = 4$ elements (♂) represents altogether different chromosome characters from the genus Pterochlorus with 8 elements.

In the genera Periphyllus and Calaphis there are species with a small *m*-element which is bivalent

CONTENTS

Introduction ... 374
Methods and materials ... 376
a. Method of study ... 376
b. Material ... 376
Observations ... 381
a. Spermatogenesis of Euceraphis betulae Koch ... 381
1. Spermatogonia ... 383
2. Spermatocytes ... 384
b. The number of chromosomes in representative species ... 385
1. Dilachnus laricis (Walker) ... 385
2. Dilachnus pinidensiflorae (Essig and Kuwana) ... 386
3. Eulachnus piniformosanus Takahashi ... 387
4. Tuberolachnus viminalis (Fonscolombe) ... 387
5. Pterochlorus tropicalis van der Goot ... 388
6. Shivaphis celti Das ... 388
7. Phyllaphis fagi (Linnaeus) ... 390

[1] A contribution from the Zoölogical Laboratory of the Imperial College of Agriculture and Forestry at Morioka, Japan. The work began at the Zoölogical Laboratory of the University of California, continued at the University of Missouri, and concluded at the Laboratory of the Morioka College of Agriculture and Forestry, Morioka, Japan, with aid from Saito Gratitude Funds, Sendai, Japan.

8. Chromaphis magnoliae Essig and Kuwana ... 391
9. Myzocallis castanae (Fitch) ... 391
10. Euceraphis betulae Koch ... 392
11. Tuberculatus quercicola (Matsumura) ... 392
12. Tuberculatus kashiwae (Matsumura) ... 393
13. Callipterus kuricola (Matsumura) ... 393
14. Therioaphis shinae Shinji ... 393
15. Calaphis betulaecolens Fitch ... 393
16. Calaphis magnolicolens Takahashi ... 395
17. Symydobius kabae (Matsumura) ... 395
18. Drepanosiphum platanoides (Schrank) ... 396
19. Drepanophis aceriifoliae Thomas ... 396
20. Chaitophorus sali-apterus Shinji ... 396
21. Chaitophorus sali-niger Shinji ... 397
22. Periphyllus aceris (Linnaeus) ... 397
23. Periphyllus koelreuteriae (Takahashi) ... 397
24. Melanoxantherium sali-japonica Shinji ... 398
25. Cavariella oenanthi Shinji ... 398
26. Carolinaia tade Shinji ... 399
27. Aphis sambuci Linnaeus ... 400
28. Acaudus itadori (Shinji) ... 400
29. Akkaia polygoni Takahashi ... 401
30. Myzus suguri Shinji ... 401
31. Amphorophora ribicola (Oesthind) ... 402
32. Amphorophora lespedezae (Essig and Kuwana) ... 402
33. Amphorophora magnoliae (Essig and Kuwana) ... 403
34. Macrosiphum gobonis Matsumura ... 403
35. Macrosiphum sonchi (Linnaeus) ... 403
36. Macrosiphum ibotum Essig and Kuwana ... 404
37. Macrosiphum cornifoliae Shinji ... 405
General considerations and remarks ... 405
1. The sex chromosome and sex ... 405
2. Evolution of the chromosomal numbers ... 411
3. On the importance of the chromosome number for systematic studies. 416
a. The genera Tuberolachnus and Pterochlorus ... 417
b. The genera Myzocallis, Callipterus, and Tuberculatus ... 417
c. The genera Euceraphis, Calaphis, Symydobius, and Yezocallis 418
d. The genera Chaitophorus and Periphyllus ... 419
Summary ... 419
Literature cited ... 420

INTRODUCTION

During the past decade cytological studies of the germ cells of aphids have brought about many far-reaching, important facts. Although nearly twenty species of aphids, including phylloxerans, have been investigated, there still remain cer-

tain paramount questions regarding the chromosomal history of aphids to be solved. To contribute as much as possible to this subject, this paper is presented. Although several important observations were made, the writer wishes to limit the scope of the present paper to only a few of them. One such problem concerns the number of sex chromosomes found in the germ cells of the aphids. Whether the number is limited to 'two' in the case of aphids, or varies with the species or genus, as in the case of reduviids studied by Payne ('15), is of much importance, for Morgan ('15), Doncaster ('20), and others all agree in stating that "there are only two sex chromosomes in the Aphids," and that 1X represents the male, and 2X the female characteristics, etc.

Another problem that demands a definite solution is the manner of synapsis and the nature of the double chromosomes of Stevens, who thinks double chromosomes represent all the chromosomes, including the sex chromosomes.

Especial consideration will, however, be given to the problem of the possible relation between numbers of chromosomes and the evolution of genera and species, leading to a more satisfactory system of classifying aphids, such as Stevens ('05) wished to have.

Before going further, I wish to express my hearty thanks to Professor Long, of the University of California, under whose untiring guidance the work was first begun in July, 1913, and continued there until September, 1915. My hearty thanks are also due to Professors Kihara and Yuasa, of the Imperial University of Kyoto, both of whom kindly gave me their valuable suggestions and criticism, besides reading through the manuscript. My special thanks must be extended to Professor Kihara for his kindness in looking over the preparations and confirming the counts of chromosomes, and also furnishing me with some photomicrographs. I am also indebted to the late Professor Lefevre, of the University of Missouri, with whom the work was continued for a year.

METHODS AND MATERIALS

a. Method of study

Most of the material studied was obtained from individuals reared on potted trees bought at nurseries and brought into the laboratory through the kindness of Professor Long, or by grants from Saito Gratitude Funds, Sendai, Japan. For the study of the female germ cells, parthenogenetic and sexual, the ovaries of adults as well as of the young of all ages were dissected out either directly in the fixing fluid or after preliminary handling in Ringer's solution.

One of the fixing reagents most generally used was Flemming's weak as well as strong solution. Carnoy's aceto-alcohol-chloroform mixture was good for the fixation of winter eggs, but in the study of the male cells it proved far inferior to Telensky's or Champy's, both of which gave as good results as Flemming's. Sections of the testis were made from 4 to 7 μ in thickness, and stained mostly with Heidenhain's iron-alum haematoxylin method, followed by either orange G, acid fuchsin, or Congo red. Flemming's triple stain was also used with fairly good results. Phospho-tungstic-acid haematoxylin was used toward the end of the last year's work. This was rather difficult to use, but in specimens properly stained I have found the very best and most beautiful chromosome figures.

b. Material

Stevens' ('05) statement that the present system of classifying aphids is unsatisfactory and therefore a new one based upon cytological study should be devised led me to the study of a great many aphids, cytologically as well as morphologically. Such investigations, however, contrary to her claim, convinced me that the present system of classification is better than, or at least as good as, the one that may be derived from cytological studies. Stevens based her dissatisfaction on the ground that no less than three different forms or species of aphids infesting the rose were placed under the name of Aphis rosae L. Had she carefully gone through the

literature on the rose aphids, she would have probably found out that there is no aphid at present that bears the name of Aphis rosae, and that several aphids infesting the rose are not called indiscriminately under the same name, but that they are known as Macrosiphum rosae, Myzaphis rosarum, Myzus persicae, Rhopalosiphum nervatum, Macrosiphum solanifoliae, Aphis gosyppii, etc. The same is true in the case of the maple aphids, etc. From what I have just said, it will become clear that the system of naming the aphids according to their host plants, as Stevens did, is much more confusing and unsatisfactory than the present system of classification based on differences and similiarities in external characters.

The species, the male germ cells of which have been studied by me, include the following thirty-seven species belonging to twenty-six different genera:

Family Aphididae

Subfamily Aphidinae
- Tribe I, Lachnini
  - Subtribe Lachnina
    - Genus 1, Dilachnus
      - Species 1, Dilachnus laricis (Walker)
      - Species 2, Dilachnus pinidensiflorae (Essig and Kuwana)
    - Genus 2, Eulachnus
      - Species 3, Eulachnus piniformosanus Takahasi
  - Subtribe Pterochlorina
    - Genus 3, Tuberolachnus
      - Species 4, Tuberolachnus viminalis (Fonscolombe).
    - Genus 4, Pterochlorus
      - Species 5, Pterochlorus tropicalis van der Goot
- Tribe II, Callipterini
  - Subtribe Phyllaphidina
    - Genus 5, Shivaphis
      - Species 6, Shivaphis celti Das
    - Genus 6, Phyllaphis
      - Species 7, Phyllaphis fagi (Linnaeus)
  - Subtribe Callipterina
    - Genus 7, Chromaphis
      - Species 8, Chromaphis magnoliae Essig and Kuwana
    - Genus 8, Myzocallis
      - Species 9, Myzocallis castanae Fitch

Genus 9, Euceraphis
Species 10, Euceraphis betulae (Koch)
Genus 10, Tuberculatus
Species 11, Tuberculatus quercicola (Matsumura)
Species 12, Tuberculatus kashiwae (Matsumura)
Genus 11, Callipterus
Species 13, Callipterus kuricola (Matsumura)
Genus 12, Therioaphis
Species 14, Therioaphis shinae Shinji
Genus 13, Calaphis
Species 15, Calaphis betulaecolens Fitch
Species 16, Calaphis magnolicolens Takahashi
Genus 14, Symydobius
Species 17, Symydobius kabae (Matsumura)
Subtribe Drepanosiphina
Genus 15, Drepanosiphum
Species 18, Drepanosiphum platanoides (Schrank)
Genus 16, Drepanaphis
Species 19, Drepanaphis acerifoliae Thomas
Subtribe Chaitophorina
Genus 17, Chaitophorus
Species 20, Chaitophorus sali-apterus Shinji
Species 21, Chaitophorus sali-niger Shinji
Genus 18, Periphyllus
Species 22, Periphyllus aceris (Linnaeus)
Species 23, Periphyllus koelreuteriae (Takahashi)
Subtribe Pterocommina
Genus 19, Melanoxantherium
Species 24, Melanoxantherium sali-japonica Shinji
Tribe Aphidini
Subtribe Aphidina
Genus 20, Cavariella
Species 25, Cavariella oenauthi (Shinji)
Genus 21, Carolinaia
Species 26, Carolinaia tade Shinji
Genus 22, Aphis
Species 27, Aphis sambuci Linnaeus
Genus 23, Acaudus
Species 28, Acaudus itadori (Shinji)
Subtribe Macrosiphina
Genus 24, Akkaia
Species 29, Akkaia polygoni Takahashi
Genus 25, Myzus
Species 30, Myzus suguri Shinji

Genus 26, Amphorophora
Species 31, Amphorophora ribicola (Oestlund)
Species 32, Amphorophora lespedezae (Essig and Kuwana)
Species 33, Amphorophora magnoliae (Essig and Kuwana)
Genus 27, Macrosiphum
Species 34, Macrosiphum gobonis Matsumura
Species 35, Macrosiphum sonchi (Linnaeus)
Species 36, Macrosiphum ibotum Essig and Kuwana
Species 37, Macrosiphum cornifoliae Shinji

A recent synopsis of the aphids enumerated above is, according to Baker, who is one of the best authorities on the subject, as follows:

Key to the tribes of the subfamily Aphidinae

1. Cornicles situated on broad flat cones . . Lachnini
   Cornicles truncate, or more or less elongate 2
2. Cornicles truncate or elongate; when elongate, the cauda knobbed, and the anal plate bilobed, or the antennae prominently hairy . . . . . Callipterini
   Cornicles not truncate, usually elongate. Cauda never knobbed. Antennae with only a few spine-like hairs . . Aphidini

Key to the subtribes of the Lachnini

1. Radial sector of fore wings curved and of moderate length. Stigma elongate . . . . . Pterochlorini
   Radial sector of fore wings short and straight, situated near the tip of the wing 2
2. Form elongate and very narrow; antennae with bristles, cornicles not hairy; eyes without ocular tubercles . . Eulachnina
   Form not elongate; cornicles on hairy cones; eyes with ocular tubercles . . . . . Lachnina

Key to the genera of the Pterochlorina

1. Abdomen with a large dorsal tubercle . . . . . Tuberolachnus
   Abdomen without a large dorsal tubercle . . . . . Pterochlorus

Key to the subtribes of the Callipterini

1. Antennae armed with rather long, prominent hairs 2
   Antennae usually only with minute, sometimes stout bristles 3
2. Cornicles cylindrical or vasiform . . . . . Pterocommina
   Cornicles truncate, enlarged at base . . . . Chaitophorina

3. Cornicles reduced to mere rings; large lateral abdominal wax plates present ..... Phyllaphidina
   Cornicles usually not reduced to mere rings; no large abdominal wax plate present 4
4. Cornicles variable, often long and somewhat swollen, Drepanaphidina
   Cornicles never long, always short and truncate ... Callipterina

Key to the genera of the Phyllaphidina

1. Anal plate deeply cleft and U-shaped ..... Shivaphis
   Anal plate somewhat bilobed, cauda rounded ..... Phyllaphis

Key to the genera of the Callipterina

1. Cauda distinctly knobbed, anal plate usually bilobed or somewhat deeply divided 3
   Cauda not distinctly knobbed, anal plate entire or almost so . 2
2. Antennae not minutely setose, sensorium at base of unguis long and narrow ..... Euceraphis
   Antennae minutely setose, sensorium at base of unguis oval or somewhat rounded ..... Symydobius
3. Anal plate deeply divided with U-shaped cleft so that the lobes appear as distinct; cauda knobbed ..... Therioaphis
   Anal plate bilobed, not deeply divided; cauda very markedly knobbed 4
4. Antennae and often the cauda with prominent hairs ... Callipterus
   Cornicles and antennae without such hairs 5
5. More or less distinct antennal tubercles present ..... Calaphis
   No distinct antennal tubercles present 6
6. Anal plate distinctly indented, sometimes almost entire; no apterous viviparous forms developed ..... Chromaphis
   Anal plate distinctly bilobed; apterous viviparous forms common 7
7. Abdomen with three pairs of long tubercles ..... Tuberculatus
   Abdomen with no such dorsal tubercles ..... Myzocallis

Key to the genera of the Drepanosiphina

1. Cornicles extremely long and swollen in the middle. Drepanosiphum
   Cornicles not reaching the tip of the abdomen..... Drepanaphis

Key to the genera of Chaitophorina

1. Cauda quite distinctly knobbed ..... Chaitophorus
   Cauda not knobbed, but rounded ..... Periphyllus

Key to the genera of the Pterocommina

1. Cornicles somewhat swollen . . . . . Pterocomma
   Cornicles cylindrical . . . . . Melanoxantherium

Key to the subtribes of Aphidini

1. Antennal tubercles distinct . . . . . Macrosiphina
   Antennae without distinct tubercles . . . . Aphidina

Key to the genera of the Aphidina

1. Hind wings with the cubitus lacking . . . . . Carolinaia
   Hind wings with both media and cubitus present 2
2. Abdomen with a dorsal projection or tubercles above cauda, Cavariella
   Abdomen without this structure 3
3. Cauda apparently absent . . . . . Acaudus
   Cauda normal . . . . . Aphis

Key to the genera of the Macrosiphina

1. Antennal tubercles with long horn-like projections . . . . Akkaia
   Antennal tubercles without large projections 2
2. Cornicles swollen . . . . . Amphorophora
   Cornicles cylindrical or tapering, scarcely swollen 3
3. Antennal tubercles strongly converging . . . . . Myzus
   Antennal tubercles distinctly diverging . . . . Macrosiphum

OBSERVATIONS

a. Spermatogenesis of Euceraphis betulae Koch (figs. 1 to 32)

This is a large form. The stem mother hatches late in February from an egg laid in the fall by an oviparous female. The newly hatched nymph is dark orange at first, on account of the presence of the yolk substances in the body. At about the time when the yolk contents of the nymph become exhausted, buds of the host plant, the birch, break out, and the insect migrates and feeds on the young leaves. When a twig infested with eggs is brought into the room and left to dry, the buds, of course, do not open, and consequently the newly hatched young die of starvation. If such twigs were placed in a tumbler containing water, or planted in a moist soil, the buds would open in the spring and the young hatch. These

would mature in from eighteen to twenty-four days, depending on the condition of the host plant and temperature, and commence to deposit young viviparous larvae, which, in turn, after a lapse of about twenty days, give rise to numerous young viviparous females, and so on. Yet this condition of parthenogenesis does not last very long. In California, New York, and Missouri, U. S. A., where I have made actual observations, the male and female forms appeared sooner or later.

For the purpose of determining the sequence of the male germ cells, several methods of experimentation were adopted. The one suggested to me by Doctor Long was the best, so far as mortality is concerned. The method is briefly as follows: by means of a hot needle a tiny drop of melted wax was placed between the wings of the adult as soon as it emerged from the final molting skin, and the insect was transferred to a fresh clean leaf, and then the whole was enclosed within a small lamp-chimney, 1 inch in diameter, with cotton plugs at both ends. The chimneys were then tied by means of a string to boughs. As soon as an adult female deposits young, she is transferred to another chimney until she gives birth to another nymph, and so on. By this method, a complete life history of the larva from the time of its birth to its maturity could be worked out. In general, it may be said that an adult female deposits, at the temperature of 70° to 80°F., one young every two hours. Since there are eight ovarioles in this species, it may be said that the depositing cycle of ovarioles in this form is sixteen hours. It follows that the period required by an egg to mature is about sixteen hours. Thus it is comparatively an easy matter to determine the sequence of the maturation figures found in different egg chambers or sperm cysts of the same, or different, ovarioles or testes, as the case may be. It is, however, difficult to determine definitely this time factor in different individuals because of variations in temperature and other environmental conditions.

The chromosome numbers of the somatic as well as the germ cells of the male and female have already been given in

my brief communication (Shinji, '18) entitled the "Chromosomes of a certain aphid, Euceraphis betulae Koch." In the present paper an attempt will be made to analyze the probable method by which the reduction in the number of chromosomes of this species may be brought about.

The male sexual organ develops in general like that of the female, the only difference being the relative size, shape, and number of the germ cells contained. There is exactly the same number of the testicular lobes as of ovarioles, at least in the case of Euceraphis betulae Koch: four on each side of the main axis of the body. The testicular lobes on each side are, however, open at the same point, forming a radial structure. Consequently, the pedicels or the sperm ducts are all very short. Another difference observable between the sexual organs of the male and the female is the number of follicular cells that invest the germ cells. These in the female organ increase in size with the growth of the egg. In the male this is not the case. Each one of the eight testicular lobes is divided into compartments, and the cells in each compartment are approximately of the same size and stage of development, suggesting that they are probably all derived from a single primordial germ cell.

1. Spermatogonia (figs. 1 to 14). The spermatogonia of Euceraphis betulae Koch are, as a rule, found in the still unborn larva. They are also found near the free end of the testicular lobes of the nymphs, until an early part of the third larval stage, but they are rare in winged adults. Since the chromosomal history of the spermatogonium is essentially the same as that of the somatic cell, only a brief account will be given here.

The resting spermatogonia are rather numerous, indicating that this stage is of considerable duration. The shape of the cell at this stage is slightly ovoid. The cytoplasm of the spermatogonia stains decidedly darker than that of the spermatocytes. No centrosomes could be detected. The nucleus contains a large chromatin nucleolus, or the karyosphere of Blackman ('05) and others. This karyosome is spherical,

and is located at one pole of the nucleus. It appears black and homogeneous in specimens stained with Heidenhain's haematoxylin. Lightly stained, it presents a vacuolated appearance (figs. 3, 4, 5), often showing within it a granular, chromatoid body. The region near the karyosome is occupied by acidophilic granules which usually radiate from the karyosome. Although no visible substance was found toward the other pole of the cell, it seems that this transparent area may probably be occupied by such a substance as the 'gel' of Gates ('15).

Following the stage described, both the cytoplasm and the karyosome become clearer. The karyosome disappears gradually and the chromatin derived mostly from it becomes scattered on an achromatic network, later being transformed into fine threads. But even when the bead-like chromosomal threads become recognizable, the karyosome can still be seen. The chromosomes gradually shorten and then finally appear as two long pairs and four shorter elements. The spermatogonial prophase, with no karyosome, is illustrated by figures 7 and 8. The two pairs of long chromosomes are autosomes.

Figures 9 to 11 represent polar views and figure 12 a lateral view of a metaphase figure. Figure 13 and 14, anaphase figures, show that there is no extrusion, or lagging behind, of any of the chromosomal parts.

2. Spermatocytes (figs. 15 to 32). During the late telophase of the last spermatogonial mitosis and the growth period of the first spermatocytes the nuclei are largely achromatic. Soon, however, chromatic granules appear on the achromatic threads, the number of which seems to equal twice that of the definitive chromosome number. As the chromatin granules increase, the nucleus begins to appear dark and continues so until the late anaphase of the first maturation division. In the following stage, a paired condition becomes more apparent among the eight doubled chromosome fibers (figs. 17 to 20).

Now, if we regard each of the paired chromosomes as representing a single spermatogonial chromosome, we should

have as many chromosomes as we see here, namely, eight pairs. Thus there is no alternative than to regard the pair as due to the splitting of the spermatogonial chromosomes. In this respect, then, my interpretation is in strict accordance with Morgan's ('15) suggestion, namely, that the chromosomes here apparently "split longitudinally, in anticipation of the second maturation division, which closely follows the first without an intermission of a resting stage."

Now let us go back to the spermatocyte stage of the birch aphid. In figures .24 and 25 there are two pairs of large chromosomes and four small ones. Thus the total number is now six. This numerical change is due to two factors: first, the space between chromosomes became obliterated, and, secondly, this obliteration was followed by a side-by-side conjugation of the larger chromosomes to form two bivalents.

As the maturation division progresses, the parts of the bivalent autosomes go to opposite poles, but the heterochromosomes lag and then are suddenly withdrawn into a single daughter cell. Those cells which receive two autosomes and four heterochromosomes undergo another mitosis before they are transformed into spermatozoa, while those which received two autosomes and no heterochromosomes degenerate in situ (figs. 29 and 30). Thus, all the spermatocytes of the second order have six chromosomes, as is illustrated by figure 31.

Descriptions and discussions on certain phases of chromosomal history are left to the time when more favorable species will be taken up.

b. The number of chromosomes in representative species

1. Dilachnus laricis (Walker) (figs. 33 to 40, 161). *Lachnus laricicolus Matsumura (1917)*. This is one of our largest species, being nearly as large as Pterochlorus tropicalis, to which it is closely related. The male germ cells are, however, very much larger than those of Pterochlorus. Figure 33 shows a polar view of the first spermatocyte. In this figure there are five chromosomes, four of which are bivalent, the remaining one univalent. This univalent, or X element,

is somewhat larger than any bivalent. In figure 36, an anaphase, the X element is much drawn out between the daughter groups, each of which consists of four chromosomes. The X chromosome is later withdrawn into one of the two daughter groups (fig. 38). This peculiar behavior of the X chromosome during the first meiotic division produces two kinds of second spermatocytes, one receiving the X chromosome and four autosomes, the other only four autosomes. The former becomes a functional spermatozoon after another division, while the latter degenerates. In the second maturation division, all five chromosomes divide equally, as figure 40 illustrates.

Counts of the chromosome number of this species are as follows:

Number of elements in spermatocyte I	4
Number of sex chromosomes in spermatocyte I	1
Total number of elements in spermatocyte I	5
Total number of chromosomes in spermatocyte II	5

One peculiar feature in the chromosomes of this species is the persistence throughout the first maturation division of a furrow that runs lengthwise of each chromosome. This may be due to the fixing fluid used, this being the only case where Gilson's mixture was employed; but I believe that this represents a rather loose approximation of the chromosomes in anticipation of the second division.

2. *Dilachnus pinidensiflorae* (*Essig and Kuwana*) (figs. 41 to 43, 162). *Lachnus pinidensiflorae Essig and Kuwana* (*1917*). *Dilachnus pinidensiflorae Baker* (*1920*). *Dilachnus pinidensiflorae Takahashi* (*1923*). This is very closely related to the preceding species, one of the conspicuous differences being the brick-brown color of the body as against dark brown in the other species, but its spermatocytes show altogether different characters. In the first spermatocyte there are eleven chromosome elements: one very large, eight medium, and two small ones. The two small ones are often seen attached to the neighboring larger one (fig. 42). The largest one is univalent, the rest bivalent (fig. 43). Thus the

diploid number of chromosomes for the male of this species is twenty-one, including a single large X chromosome. The X chromosome lags behind the others during the anaphase, and finally goes entire into one second spermatocyte. One second spermatocyte has ten chromosomes plus an X; the other, ten autosomes and no X. The former alone produce functional spermatozoa after another of homotypic division.

3. Eulachnus piniformosanus Takahashi (figs. 44 to 47, 163). *Eulachnus piniformosanus Takahashi* (*1922*). This is a much-elongated form infesting pine needles.

In the first spermatocyte (figs. 44 and 163) there are one oblong, or often rod-shaped, large univalent and six ovoid smaller bivalent elements, or $6_{II} + X = 13$ chromosomes. Although the number of autosomes found in this species is just three times as many as those found in Dilachnus laricis, the present case cannot be taken for one of hexaploidy for the reason that the mass of all the chromosomes taken together does not exceed that of Lachnus laricis. On the contrary, I am strongly of the opinion that the chromosome complements of this species may result from the transverse division of the autosomes found in such a case as Dilachnus laricis.

4. Tuberolachnus viminalis (*Fonscolombe*) (figs. 48 to 53, 164 and 165). *Aphis viminalis Boyer de Fonscolombe* (*1841*). *Lachnus viminalis Buckton* (*1880*). *Tuberolachnus viminalis Mordwilko* (*1908*). *Lachnus viminalis Matsumura* (*1917*). *Tuberolachnus viminalis Essig* (*1912*). *Tuberolachnus viminalis Das* (*1918*). *Pterochlorus viminalis Baker* (*1920*). *Tuberolachnus viminalis Takahashi* (*1922*). This is the largest green aphid found in this country, but the male germ cells are rather small compared with those of Dilachnus laricis, which suggest an affinity rather to Pterochlorus tropicalis.

The equatorial plates of the first spermatocyte show four chromosomes, three of nearly equal size and the fourth much smaller. Since the smallest element is always bivalent (figs. 49 and 165), it cannot be the X chromosome. In fact, the X element in this case is the largest of the four if a gradation

can be detected among them. It must be mentioned here that the smallest bivalent element found in this species might, in all probability, have been produced by a cross division of one of the two remaining autosomes, and is, therefore, comparable not only in its origin, but in its size and behavior to the microchromosomes found in Metapodius by Wilson ('09).

5. Pterochlorus tropicalis van der Goot (figs. 54 to 57, 166 to 168). *Pterochlorus tropicalis van der Goot* (*1913*). This is one of the largest aphids found in this country. The somatic and germ cells of the females are very large, but the germ cells of the male are comparatively small, being no larger than those of our mid-sized Myzocallis.

The equatorial plate, especially its lateral view, often presents a peculiar chromosome (figs. 54, 55, 166, and 167). This chromosome I once took for a single chromosome showing its transverse as well as longitudinal furrows. A careful study, however, convinced me that this triangular element is, in reality, composed of four small elements (fig. 56). Thus, the chromosome number in the spermatocyte I is eight. Four large elements are distinctly double. Of the four small elements, two are bivalent, but the status of the other two is not very clear. The difference between this species and Tuberolachnus viminalis is marked, for one has four and the other eight elements. These two species have long been regarded as belonging to two genera, but recently have been placed in a single genus, Pterochlorus, by Baker ('20). If the number of chromosomes has any generic significance, they should again be separated. Therefore I have reverted to the use of the old generic name.

6. Shivaphis celti Das (figs. 58 to 60, 169). *Shivaphis celti Das* (*1917*). *Chromaphis celticolens Essig and Kuwana* (*1918*). *Phyllaphis celticolens Takahashi* (*1917*). *Shivaphis celti Baker* (*1920*). *Shivaphis celticolens Takahashi* (*1924*). *Shivaphis celti Takahashi* (*1925*). The number of chromosome elements in this species is three, as in the case of Aphis saliceti studied by von Baehr ('09) and Liosomaphis (Aphis) berberides studied by Morgan ('15). In figures 59 or 169, of

the first maturation division, we can detect a slight difference in shape and size of the two bivalent autosomes, one pair being slightly shorter than the other. The sex chromosome is not the largest, but the smallest of the three elements. This smallest one has no mate, and so goes undivided into one of the daughter cells, resulting in the formation of two kinds of second spermatocytes. One kind later degenerates without further division, while the other, which receives the sex chromosome and two autosomes, undergoes another division, producing two similar spermatids which elongate to form two functional spermatozoa as in the case of other aphids studied.

This species and such other species of the subtribe Phyllaphidina are of much theoretical importance in the evolution of Aphididae, for it is through this species and Tamalia (Phyllaphis) coweni studied by Morgan ('15) that a phylogenetic connection between the subtribe Callipterina belonging to the tribe Callipterini to which this species also belongs, on the one hand, and such other tribes as Lachnini and Aphidini on the other, is established. When the chromosomes of this species are compared with those of other species belonging to different tribes, it becomes clear that it represents one of the most primitive character, for its autosomes are all of elongated type, which conjugate loosely during the first meiotic division as against the oval shape and closer conjugation type of other subtribes.

In this connection it is of much interest to note that this is one of the three rare species studied, in which the young and adult individuals secrete a flocculent substance, the other two species being Euceraphis betulae and Phyllaphis fagi. Whether this habit of secretion is correlated with a certain characteristic of chromosomes or not, cannot easily be determined. Although it is but a conjecture, yet it is of interest to mention that the X chromosome in Shivaphis or Euceraphis is much smaller than autosomes, while that of Phyllaphis is the largest, and that the autosomes of the two first named are of the elongate rod type as against the oblong or oval type found in Phyllaphis. The only common character

for the three woolly aphids, which is lacking in other species, is the presence in each of the bivalent autosome elements of a wide furrow. Thus, should there be any chromosome character that correlates with the secretion of flocculence, that character may probably be the loose pairing of the homologous chromosomes.

7. Phyllaphis fagi (*Linnaeus*) (figs. 61 to 64, 170). *Aphis fagi Linnaeus* (*1767*). *Lachnus fagi Burmeister* (*1767*). *Phyllaphis fagi Koch* (*1857*). *Phyllaphis fagifoliae Takahashi* (*1923*)? Although Takahashi has recently described a new woolly aphid from this country under a new name of Phyllaphis fagifoliae, the description, especially of the male, of Phyllaphis fagi given by van der Goot so well fits my material that I regard the species as the same as the one found in Europe. In spite of the smallness of the body, this aphid has good-sized germ cells.

The first spermatocytes all show thirteen elements, including one univalent. This univalent element, or X chromosome, is four or five times as long as the largest of the autosomes and lies usually in the center of the group. Among the remaining twelve, some are disproportionately small (figs. 61, 63, and 170). The lagging of the X chromosome during the anaphase and its final withdrawal into one of the second spermatocytes are characteristically present.

It is of much interest to note that here also we have a probable case of the multiplication of chromosomes in the two species belonging to the same subtribe. In the case of the subtribe Lachnina we had chromosome numbers of $4_{II} + X = 9$, $6_{II} + X = 13$, and $10_{II} + X = 21$. Here we have $2_{II} + X = 5$ and $12_{II} + X = 25$, or a species having five times as many chromosomes as its near ally. The probable cause of this multiplicity of chromosomes will be discussed later, but it must be mentioned here that this case cannot be compared with the so-called polyploidy found in hybrids or mutants of other animals and plants, for the sizes of chromosomes found do not warrant such an assumption. The more satisfactory explanation of this chromosome complex will be that here,

again, the four original autosomes of such a type as that found in Shivaphis celti have undergone repeated transverse divisions to produce twenty-four chromosomes, for the amount of all the autosomes seems to be equal in this case to that of the two long bivalent elements found in the case of Shivaphis celti. That all the chromosomes found in this species may probably be derived directly from those of Shivaphis type rather than those of Aphis type studied by von Baehr ('09) finds another corroboration in the fact that this species, like the preceding one, has the habit of secreting a flocculent substance—very rare instances found among the species of the subfamily Aphidinae.

8. Chromaphis magnoliae Essig and Kuwana (figs. 65 to 67, 171 and 172). *Chromaphis magnoliae Essig and Kuwana (1918)*. This is one of the middle-sized aphids infesting Magnolia kobus, with medium-sized germ cells. Semipolar views of the equatorial plate of the first maturation division (figs. 65, 171, 172) show four elements, three large and one very small. The lateral view of a similar plate shows that the smallest and two of the three large elements are, without exception, bivalent, but that one of the largest elements is surely univalent. Since the three larger elements are approximately of the same size, it often becomes difficult to identify the unpaired element, but it must be mentioned that, whenever any difference in size can be detected among the three, the univalent element is always the largest of them all (fig. 66).

9. Myzocallis castanae (Fitch) (figs. 68 to 70). *Callipterus castanae Buckton (1883)?* Although a great many specimens of this species were collected and sectioned, they were worthless, partly because of old age and partly because of bad fixation. Only one specimen showed a few cysts containing first and second spermatocytes. The polar views of the metaphase of the first spermatocyte show seven chromosome elements: one very large rod-shaped, five medium spherical, and a single tiny round one (figs. 68 and 69). Figure 70 represents a lateral view of such a spermatocyte. In this and

other lateral views the largest element is found to be univalent, the rest bivalent. Scanty as the foregoing observations on the spermatogenesis of the chestnut aphid are, they indicate that the spermatocyte chromosome number in this species is $6_{II} + X = 13$, and that the largest chromosome represents the X.

It is of much interest to note that there is in this species a tiny bivalent chromosome comparable to the microchromosomes of Metapodius studied by Wilson ('09). Although I have no direct evidence, yet I am strongly of the opinion that this tiny element may, like the microchromosome of Metapodius terminalis, be the product of a cross division of a larger autosomal element.

10. Euceraphis betulae Koch (figs. 1 to 32 and 173 and 174). *Callipterus betulae Koch (1855). Callipterus betulicola Buckton (1885). Euceraphis betulae Baker (1920).* The spermatogenesis of this species has been described at the beginning of the present treatise. But, since this was the only species of aphids with four X chromosomes, our counts of the chromosome number, especially those of X chromosomes, were often questioned. The fact, however, that the spermatogonial metaphase plates always show eight chromosomes, four longer and somewhat curved ones and three shorter ones of graded sizes in addition to a single oval or spherical one, is, we believe, a positive proof of the existence of four unpaired or X chromosomes.

11. Tuberculatus quercicola (Matsumura) (figs. 71 and 72). *Acanthocallis quercicola Matsumura (1917). Myzocallis quercicola Takahashi (1923).* This is one of the Tuberculatus infesting oaks—Quercus dentata and Quercus serrata. The male germ cells are medium in size, and their chromosomes rather thick. Figure 71 represents a polar view of the metaphase figure of the first spermatocyte. In this figure there are seven chromosomes. The lateral view of the metaphase figure, such as the one represented by figure 72, shows that the largest one of the elements alone is univalent. Thus the chromosome characters of this species are almost the same as those of Tuberculatus kashiwae.

12. Tuberculatus kashiwae (*Matsumura*) (figs. 73 and 74). *Acanthocallis kashiwae Matsumura* (*1917*). *Myzocallis kashiwae Baker* (*1920*). This species, too, has seven elements in the first spermatocytes (fig. 73). Of the seven elements, the X, or univalent element, is nearly twice as large as the largest autosome. The smallest element, which corresponds with the microchromosome of Myzocallis castanae, is much larger, being almost half as large as the next smallest one.

13. Callipterus kuricola (*Matsumura*) (figs. 75 to 77, 175). *Nippocallis kuricola Matsumura* (*1917*). *Callipterus kuricola Baker* (*1920*). This is a rather dark-looking small aphid infesting the underside of leaves of the chestnut. The number of chromosomes found in the spermatocytes is the same as that found in Tuberculatus (figs. 75 and 175). One of the seven elements seen on the equatorial plate of the first spermatocyte is univalent, the rest bivalent. The univalent element is always the largest, and, in most cases, very conspicuous, owing to its slanted position on the equatorial plate. As there is no very small element present, all species of the genus Callipterus can be told from those of the genus Myzocallis. The difference between this species and those of the genus Tuberculatus is often difficult to make out, but, in general, the X element of this species seems to be much larger than that of the genus Tuberculatus.

14. Therioaphis shinae Shinji (figs. 78 and 79). *Therioaphis shinae Shinji* (*1924*). This is one of the beautiful Callipterini, resembling in many respects a Callipterus or a Myzocallis. The number of chromosomes found in the male cells is exactly the same as that of the two other genera mentioned. The polar view of the equatorial plate of the first spermatocyte shows seven elements (fig. 78), and the lateral view six bivalent and one univalent. The largest is always univalent.

15. Calaphis betulaecolens Fitch (figs. 80 to 82, 176 and 177). *Calaphis betulaecolens Fitch* (*1856*). Excellent preparations of this species were found among the material collected in California, U.S.A., and fixed in either Flemming's strong mixture or Carnoy's aceto-alcohol-chloroform solution.

A feature observed in the first spermatocyte is an abundance of such figures as illustrated in figure 80. At first I thought that these might represent the contraction, or synizesis, stage so commonly observed in other animals, but later came to think that these may probably be due to poor fixation.

A view of a first spermatocyte prophase is shown in figure 81. Figures 176 and 177 show nine elements. A lateral view of the metaphase of the first maturation division shows the exact identity of the unpaired chromosome (fig. 82). Thus, in Calaphis betulaecolens there is only one heterochromosome—the largest one. Figure 82 shows also the fact that each one of the paired chromosomes is connected with an independent fiber running from the equatorial plate to one of the poles of the cell, while the univalent is found on a single fiber running from one pole of the cell to the other. Thus, it happens, that halves of paired chromosomes go to opposite poles, but the sex chromosome becomes pulled out to form a lagging chromosome, which later is pulled entirely into one daughter cell, as in the case of the other aphids studied. The result of this peculiar behavior of the heterochromosome is this: one of the daughter cells receives eight autosomes and one heterochromosome, and the other eight autosomes only. The cells of the latter kind later degenerate, while those of the former kind become functional spermatozoa after another mitosis.

Stevens' ('06) figure 43, which represents a lateral view of the metaphase of the first maturation division of male germ cells of the paper birch aphid, resembles my figures 81 and 82 in the number and relative size of the chromosomes. Although in her figure the heterochromosome is found in the center of the chromosomal ring, I am inclined to think that her birch aphid and this Calaphis may prove to be the same species.

The genus Calaphis with $8_{II} \pm X = 17$ chromosomes ought to be regarded as a much higher form in the evolutional scale than other genera having $6_{II} + X = 13$ chromosomes.

16. Calaphis magnolicolens Takahashi (figs. 83 to 86). *Calaphis magnolicolens Takahashi* (*1919 a*). This is a light green form infesting the underside, and especially the ribs, of Magnolia hypoleuca S. and Z. The equatorial plate of the first spermatocyte of this species resembles in general appearance that of the preceding species, but the number of chromosomes differs in that there are ten instead of nine (fig. 83). The X element in this species is, too, the largest. Of the remaining nine elements, eight are of such a nature that they form a series of graded elements in size, but the smallest is decidedly smaller than the smallest of the eight. So, I cannot but think that this tiny element, which is comparable to the micro-element found in the case of Myzocallis castanae, is a recent division product of one of the eight autosomes found in the case of Calaphis betulaecolens. In this account of the division of an element alone we find a satisfactory explanation for the presence of two different sets of chromosomes in the species of the same genus.

17. Symydobius kabae (*Matsumura*) (figs. 87 to 89). *Yezocallis kabae Matsumura* (*1917*). *Euceraphis japonica Essig and Kuwana* (*1918*). This species presents a very marked resemblance to Phyllaphis fagi (Linnaeus) in the number of chromosomes as well as the general appearance of the spermatocytes. The cell is, however, somewhat smaller than those of Phyllaphis.

The equatorial plate of the first spermatocyte shows thirteen elements; the largest is univalent, the others bivalent. The bivalents are of graded sizes and various shapes, but only the X chromosome is rod-shaped. It is usually found in the center of the ring of other chromosomes (figs. 87 and 88).

The existence of the same number of chromosomes of similar shape and size in two species of different subtribes points to a probability that there may exist in the chromosomes of the aphids many points of weakness where the fragmentation may take place, as in the case of grasshoppers studied by Carothers ('17).

18. Drepanosiphum platanoides (Schrank) (figs. 90, 91, and 178). *Aphis platanoides Schrank (1801). Aphis platanoides Kaltenbach (1843). Aphis platanoides Walker (1870). Drepanosiphum platanoides Koch (1855). Drepanosiphum platanoides van der Goot (1913).* In the equatorial plate of the first spermatocyte there are fifteen elements. One of the striking features observable during the metaphase of the first maturation division is the presence of three small chromosomes usually in the interior, yet often along the margin of the chromosome plate (fig. 90). These elements often lie at the corners of a triangle. The remaining twelve elements are of varying sizes. A lateral view of the equatorial plate (fig. 91) shows that fourteen of the fifteen elements are bivalent; the remaining one, the largest, is univalent.

Stevens ('06, '09) presents a few figures illustrating the presence in the maple aphis of a sex chromosome. Although she did not mention the scientific name of the species studied, her explanation of the figures and also her figure 48 in her 1909 paper suggest the identity of her species with mine, namely, Drepanosiphum platanoides Schrank.

19. Drepanaphis acerifoliae Thomas (figs. 92 to 95). *Drepanosiphum acerifoliae Thomas (1880).* This species was collected at Berkeley, California, U.S.A. A polar view of the equatorial plate of the first meiosis is shown by figure 93. In this figure there are nineteen elements, sixteen being almost similar in shape and size. Although the anaphase figures show the presence of a rather large univalent element, its identity is rather difficult to make out on account of the similarity existing among the larger elements.

20. Chaitophorus sali-apterus Shinji (figs. 96 and 97). *Chaitophorus sali-apterus Shinji (1923).* This is the smallest green aphid found in this country, and its germ cells are also relatively small. There are seven elements in all: one large univalent, or X, and six bivalent elements of varying sizes (fig. 96). This univalent element becomes much drawn out between the two daughter cells during the anaphase, but at the close of that phase it is suddenly taken into one second

spermatocyte. Thus one second spermatocyte has an X element and six autosomes; the other, six autosomes and no X chromosome. The former alone produces the functional spermatozoa after another division of the homotypic type. So, the number and behavior of the chromosomes of this species are exactly like those found in the case of the genera Myzocallis, Tuberculatus, and Callipterus of the same tribe.

21. Chaitophorus sali-niger Shinji (figs. 98 to 100). *Dichaitophorus sali-niger Shinji* (*1923*). This is a small blackish aphid infesting the underside of leaves of the willow. The male cells show the same number of chromosomes as the preceding species. There are seven elements present (figs. 98 and 99). Of this number, one is very much larger than the others and is univalent (fig. 100).

22. Periphyllus aceris (*Linnaeus*) (figs. 101 to 103, 180). *Aphis aceris Linnaeus* (*1767*). *Aphis aceris Fabricius* (*1794*). *Aphis aceris Kaltenbach* (*1843*). *Chaitophorus aceris Koch* (*1855*). *Chaitophorus aceris Buckton* (*1883*). *Chaitophorus aceris Matsumura* (*1918*). *Chaitophorus japonica Essig and Kuwana* (*1918*). *Chaitophorinella aceris van der Goot* (*1915*). This, and P. koelreuteriae Takahashi described below, had long been placed under the genus Chaitophorus, and it is but very recently that the new genus Periphyllus was erected for them. The important characteristics common to the two genera are the shape of the cornicles and the presence in one of a dimorphic form, which is wanting in the other. The chromosome characters of these two genera are also very different. Here, in this species, we have ten elements, including one univalent, or $9_{II} + X = 19$ chromosomes (figs. 101 and 180). The univalent, or X element, is the largest of all, the rest being, with the exception of a single tiny element, of graded sizes. This single minute element is, like that found in Myzocallis castanae, usually found close by, but often attached to, one of the remaining elements, suggesting its probable new origin.

23. Periphyllus koelreuteriae (*Takahashi*) (figs. 104, 105, and 181). *Periphyllus koelreuteriae Takahashi* (*1919 a*).

This species is a very close relative of the preceding species. The general arrangement, shape, size, and number of the chromosomes are almost identical in the two species (figs. 104 and 181). The largest one is univalent, the rest being bivalent. In this case, again, we found one small element comparable to *m*-chromosomes of Metapodius studied by Wilson ('09) or that of Bombyx mori studied by Kawaguchi ('23).

24. Melanoxantherium sali-japonica Shinji (figs. 106 to 111). *Melanoxantherium sali-japonica Shinji* (*1924*). This is one of the two aphids infesting trunks of the willow in this country, the other being Tuberolachnus viminalis. Its cells and their chromosomes resemble those of Tuberolachnus and the number of chromosomes is the same. In figure 107 there are seen three large elements besides a single small one, or four in all. Figure 106, a first spermatocyte in late prophase, and figure 108, a lateral view of the first spermatocyte, show three bivalents, including the very small one and a large univalent X chromosome. The existence of species possessing the same number of chromosomes in each of the main tribes suggests that the evolution of aphids proceeds, not at random, but along a definite path in each of the main division of tribes. Consequently, it may be assumed that in each chromosome of aphids there are certain points which may be places of fragmentation.

Baker regards the Pterocommina, to which this species and Melanoxantherium (Melanoxanthus) salicis (studied and reported by Tannreuther, '07), belong, as much higher forms than the subtribe Callipterina. The result shown by the number of chromosomes does not agree with his assumption, for the largest number of chromosomes found in Pterocommina is $3_{II} + X = 7$ chromosomes, while in the Callipterini the count runs as high as $12_{II} + X = 25$ chromosomes, represented by Symydobius kabae, or $14_{II} + X = 29$, represented by Drepanosiphum platanoides.

25. Cavariella oenauthi Shinji (figs. 112 to 117, 179). *Hydronaphis oenauthi Shinji* (*1922*). This is a rather small, pale or dark green aphid infesting roots, stems, and leaves

of Oenauthe stolonifera DC. The number of chromosomes is the same in the root forms and leaf-infesting individuals. The polar view of the equatorial plate of the first spermatocyte division shows four elements as in the case of Tuberolachnus, Chromaphis, and Melanoxantherium (figs. 113 and 179). Of the four elements, three are of nearly equal size, all of them being oblong, but the fourth element is very small and round in shape. That the X or univalent element is one of the larger ones can be learned from such figures as figures 112 and 114, the former representing a prophase condition and the latter a lateral view of the equatorial plate of the first maturation division.

26. Carolinaia tade Shinji (figs. 118 to 124, 182 to 184). *Carolinaia tade Shinji* (*1928*). The adult forms of this species very closely resemble Rhopalosiphum and its allied genera, Amphorophora and Macrosiphum, both of which belonging to the tribe Aphidina of Baker ('20). Both the size of the male germ cells and their chromosomes are very much larger than those of the allied species I have examined. In these respects, this species may be said to be the best material for the study of chromosomes of aphids.

In spermatocyte I, five elements are seen (figs. 119 and 182). Of this number, one is unpaired, the other four being bivalent. From the beginning of the anaphase on, the largest unpaired chromosome lags behind the others, but later on, it goes into one of the daughter cells. Two kinds of daughter cells are thus formed: one with four autosomes and the unpaired chromosome and the other with four autosomes only (fig. 122). Thus the behavior of the unpaired chromosome in this species is exactly like that of others studied. Whether in all the species of Carolinaia the largest one will be the X chromosome or not cannot be stated as yet. The genus Carolinaia alone, in the tribe of Aphidini, possesses five elements or nine chromosomes in the male cells. In this regard alone this species and in all probability the genus Carolinaia differs from its near allies, Acaudus, Amphorophora, and Macrosiphum, all of which have either six or seven elements, including the X chromosome.

27. Aphis sambuci Linnaeus (figs. 125, 126, and 185). *Aphis sambuci Linnaeus (1767). Aphis sambuci Kaltenbach (1843). Aphis sambuci Koch (1853). Aphis sambuci Buckton (1877). Aphis sambuci Matsumura (1917). Aphis sambucifoliae Swain (1919). Aphis sambuci Takahashi (1923).* This is one of the middle-sized aphids commonly found on leaves as well as tender shoots of the elder all over the world. In spite of its body size, the male germ cells are rather small. The number of chromosomes is $5_{II} + X = 11$, as in Acaudus itadori (figs. 125 and 185). The largest is the X chromosome. But as the chromosome elements are all of a graded size, there is no difficulty in distinguishing this species from Acaudus itadori, in which there are two small elements beside four larger ones, as we shall presently show.

The chromosome number found in this species exactly agrees with that found in Aphis on the golden-rod studied by Stevens ('09), but differs from that found in Aphis saliceti studied by von Baehr ('09), or of Aphis oenotherae reported by Stevens ('05), for von Baehr found $2_{II} + X = 5$ and Stevens $4_{II} + X = 9$ in their specimens.

28. Acaudus itadori (Shinji) (figs. 127, 128, and 186). *Neolachnaphis itadori Shinji (1923).* This species, which infests Polygonum Reynoutria, is of moderate size, but the germ cells are smaller than those of closely related species. The number of elements found in spermatocyte I is six: four uniformly large and two small, the latter being but one-quarter as large as the former (figs. 127 and 186). The behavior of the unpaired chromosome is similar to that of the species previously described. Figure 127 shows one of the polar views of the first maturation division with six elements. The lateral view of a similar equatorial plate is shown in figure 128. In this figure the X chromosome is the largest one of the six elements.

As we shall see, species with six chromosome elements, or $5_{II} + X = 11$ chromosomes, are also found in such genera as Akkaia, Aphis, Amphorophora, and Macrosiphum, belonging to different subtribes. But the present species, and all others of the genus Acaudus, will, in all probability, be dis-

tinguished from allied species or genera by the presence of two smaller elements, the size of which is no bigger than a quarter part of the next smallest element in diameter.

29. Akkaia polygoni Takahashi (figs. 129 to 131). *Akkaia polygoni Takahashi* (*1917 b*). *Akkaia polygoni Takahashi* (*1923*). One of the peculiar characteristics of this species is the possession on the antennal tubercle of a long horn-like projection directed inward. Otherwise, this species, especially the sexual forms, may fairly be regarded as an Amphorophora, or Macrosiphum. The number of elements is six, including a single univalent (fig. 129). Thus the number of chromosomes shows a close affinity of this species to others belonging to several genera of the tribe Aphidinia such as Macrosiphum, Amphorophora, Acaudus, and Aphis. Although this species resembles Acaudus itadori in the possession of two small bivalent elements, the two may be distinguished apart by the relative sizes of the two small elements. The small elements in this species are one-eighth of the size of the next larger ones, while those of Acaudus are not so small as one-quarter of any of the rest of the four elements.

30. Myzus suguri Shinji (figs. 132 and 133). *Myzus ribis Shinji* (*1924*). *Myzus suguri Shinji* (*1928*). This green aphid, infesting leaves and tender shoots of the wild as well as cultivated gooseberry, is one of the two aphids that regularly produce sexual forms during the summer months July and August at Berkeley, California, U.S.A., the other being Amphorophora ribicola. Another peculiar habit of this species is found in its egg laying. The so-called winter eggs, which are laid during the summer, are found, without exception, on the extreme ends of thorns of the host plant.

The number of chromosomes of this species, six, is the same as that of Amphorophora ribicola, the largest being univalent. The difference between this species and Amphorophora seems to be in the relative size of the chromosomes, for in this species there is only one very small element as against two of them in Amphorophora.

31. Amphorophora ribicola (*Oestlund*) (figs. 134 to 145). *Nectarosiphum ribicola Oestlund* (*1887*). *Amphorophora ribicola Baker* (*1920*). The number of the spermatogonial as well as the spermatocyte chromosomes in this species is eleven, as in Acaudus itadori and Aphis sambuci. During the growth period of the first spermatocyte each one of the eleven chromosomes shows a paired or split condition, with a furrow running lengthwise of the chromosome threads. In fact, this furrow can be detected even during the prophase of the spermatocyte of the second order, but not after the anaphase, suggesting that this separation is a preparation for the second division that takes place without a definite resting period after the first. No contraction to a pole of the chromosomes is observed, but the nuclear space becomes gradually clearer and at the same time the diplotene threads shorten. As the result of this shortening and coiling of the diplotene chromosomes, a sort of tetrad is formed, the number being six (fig. 136). What we soon see on the equatorial plate of the first division are these six elements, the largest being univalent, the rest being bivalent (fig. 138). During the anaphase this univalent element becomes stretched out between the two daughter nuclei, forming the so-called lagging chromosome of Stevens ('05, '06). The behavior of this X chromosome is very interesting. As figures 139 to 142 illustrate, it often appears as if it were actually pulled apart at the middle, or it is often so situated that its connection with the two daughter nuclei has practically been cut off. The condition that led Tannreuther ('07) and Stevens ('05) at first to believe in the absence of X chromosomes in aphids might have been some such behavior of the X element. In spite of such variations during the anaphase, it goes, without exception, into one of the two daughter cells producing, as usual, two types of second spermatocytes, only one of which is functional. Thus all the fertilized eggs and the larvae that develop from them are of one sex, the female with twelve chromosomes.

32. Amphorophora lespedezae (*Essig and Kuwana*) (figs. 146 to 149). *Rhopalosiphum lespedezae Essig and Kuwana*

(*1918*). So far as the number of chromosomes is concerned, there is no difference between this species and species of Amphorophora, Aphis, Myzus, Acaudus, and some of Macrosiphum. In the equatorial plate of the first spermatocyte there are six elements of graded sizes. Of this number, one is univalent, the remaining five bivalent (figs. 147 and 148).

33. Amphorophora magnoliae (*Essig and Kuwana*) (figs. 150, 151, and 187). *Rhopalosiphum magnoliae Essig and Kuwana* (*1918*). *Rhopalosiphum sambucicola Takahashi* (*1918*). *Rhopalosiphum sambucicola Takahashi* (*1919*). *Amphorophora magnoliae Takahashi* (*1923*). Figures 150 and 181, which represent two equatorial plates of the first spermatocytes, show six chromosome elements of graded sizes. The largest is univalent, the rest being bivalent. The difference between this species and those of such other genera as Acaudus or Aphis is obvious; in this species there is only one small bivalent element comparable to the microchromosomes of Wilson ('09) as against two in the species belonging to the other two genera just mentioned.

34. Macrosiphum gobonis Matsumura (figs. 152 and 153). *Macrosiphum gobonis Matsumura* (*1917*). *Macrosiphum gobonis Takahashi* (*1922*). This is a common black louse infesting Arctium lappa in Japan, Formosa, Korea, and China. The body is rather large, measuring in some cases as large as 2.8 mm. in length and 1.6 to 1.8 mm. in width, but its male germ cells are relatively small as compared with those of Macrosiphum ibotum and Macrosiphum cornifoliae, both of which are much smaller than this species.

The equatorial plates of the first spermatocytes invariably show seven chromosome elements, the largest one of which is univalent, the rest being bivalent (figs. 152 and 153).

35. Macrosiphum sonchi (*Linnaeus*) (figs. 154 to 156, 188). *Aphis sonchi Linnaeus* (*1767*). *Aphis sonchi Fabricius* (*1794*). *Aphis sonchi Schrank* (*1801*). *Aphis sonchi Kaltenbach* (*1843*). *Aphis campanulae Kaltenbach* (*1843*). *Siphonophora alliariae no. 1 Koch* (*1855*). *Siphonophora lactucae Koch* (*1855*). *Macrosiphum sonchi van der Goot* (*1915*).

Macrosiphum sonchi Matsumura (1917). Macrosiphum formosanus Takahashi (1922)? This is one of the most common aphids in Europe and Asia. Although the color of the body is different from the preceding species, being reddish brown, other characters resemble very much those of Macrosiphum gobonis. The chromosome characters are also similar. The equatorial plate of the first spermatocyte (fig. 154) shows six elements of nearly graded sizes. One element, which is found in the center of the equatorial plate, is peculiar in seeming to be composed of two independent elements, one very large and the other very small (fig. 188). This element may possibly represent a single element fragmenting into two. This peculiar-shaped univalent element lags behind the others during the anaphase and remains undivided. Those daughter spermatocytes which failed to receive the X chromosome degenerate in situ without undergoing another division. So all the functional spermatozoa are produced from the same kind of second spermatocytes as in all other species of aphids studied.

36. Macrosiphum ibotum Essig and Kuwana (figs. 157 and 158). *Macrosiphum ibotum Essig and Kuwana (1918).* This species produces sexual forms late in autumn. Indeed, the material could be obtained as late as December 10th. In one case where the potted host plant was kept under a shed, it has kept on producing the parthenogenetic individuals throughout the year.

Figure 157 shows a polar view of the equatorial plates in metaphase with seven chromosomes of graded sizes. A lateral view (fig. 158) shows that the largest is univalent, the remaining six being bivalent. The number of chromosomes found in this species is, then, the same as that of the genera Eulachnus in the tribe Lachnini, Myzocallis, Tuberculatus, Callipterus, Therioaphis, and Chaitophorus of the tribe Callipterini. The existence in each of the three main tribes of the subfamily Aphidinae of genera with the same number of chromosomes, namely, $6_{II} + X = 13$, clearly suggests that the evolution of aphids has taken place along the paths definitely destined for all the tribes.

37. Macrosiphum cornifoliae Shinji (figs. 159, 160, and 189). *Macrosiphum cornifoliae Shinji* (*1924*). This is a moderate-sized species. As the bodies of the nymphs of all stages are transparently light green, the cells, when stained with haematoxylin, give very definite figures. In fact, this was the only specimen permitting the study of entire cells with perfect success. In both sectioned material and that stained with aceto-carmine the equatorial plate of the first spermatocyte showed seven elements. One is large, being more than twice the size of the next largest ones, which are two in number. The remaining four can be grouped into two sizes of two each (fig. 159). The largest chromosome is univalent (fig. 160). The history of the chromosomes in meiosis is the same as that of previously described species.

Should the chromosomes of the female germ cell behave in the manner described for other forms of aphids by von Baehr ('09), Stevens ('09), and Morgan ('15), we would expect the same number of chromosomes as in the functional male spermatozoa, i.e., seven in all the matured egg cells. Should this inference be correct, we would have altogether fourteen chromosomes in the fertilized eggs, and consequently in all the cells of the parthenogenetic as well as oviparous females.

GENERAL CONSIDERATIONS AND REMARKS

1. The sex chromosome and sex

Much of the recent cytological literature has, in some way or other, to do with the so-called sex chromosome. This peculiar chromosome has been known as 'chromatin nucleolus' (Montgomery, '98, '06, '11), 'accessory chromosome' (McClung, '99, '02; Stevens, '05, and others), 'odd chromosome' (Stevens, '05), 'lagging chromosome' (Stevens, '05), 'idiochromosome' (Wilson, '00, '01, '02, '03), 'X chromosome' (Morgan, '09), 'sex chromosome' (Hegner, '15; Morgan, '15, and others). It was first discovered by Henking ('90) in the spermatocytes of a hemipteran insect, Pyrrochoris apterus. He states that in his material one of the 'chromatin elements' passed undivided into one of the daughter cells (spermatids)

during the second maturation division so that there resulted two kinds of spermatids with respect to the number of chromosomes, one receiving one more chromosome than its sister cell.

The discovery of this chromosome is of much importance, at least, in two ways: in the first place, it revolutionized the then prevalent idea that the number of chromosomes in the cells of an individual of a species is a constant, and, in the second place, it paved the way for the solution of the problem of sex determination. Henking's observation was confirmed by Paulmier ('96) in the case of Anasa, another heteropteran. The significance of this chromosome, however, remained in question until McClung ('01), who had also found a similar body in the spermatocytes of Xiphidium, an orthopteran, came forward with the suggestion that this might be a sex determinant. Since then odd chromosomes have been discovered in a large number of animals belonging to many different phyla. In all of these cases it has fully been demonstrated that they are intimately associated with the phenomenon of sex determination: those individuals with 2X, or, more correctly, 2 (X), where X represents any definite number, were found to be females, whereas those which contained 1 (X), also representing a definite number, all turned out to be the males. Up to the time when my first work on the chromosomes of aphids was published, the known cases of the chromosome number in the family Aphididae were as follows:

Name of species	*Haploid number* ♂ ♀	*Diploid number* ♂ ♀	*Name of authority*
Aphis saliceti	2, 3	5, 6	von Baehr, '09
Aphis salicis	3, 3	6, 6	Tannreuther, '07
(Melanoxanthus) salicis	3, 3	6, 6	Tannreuther, '07
(Phyllaphis) coweni	2, 3	5, 6	Morgan, '15
Pemphigus pyriformis	(9, 10)	(19) 20	von Baehr, '09
Pemphigus spirotheca	(9, 10)	(19) 20	von Baehr, '09
Aphis ('milkweed, black')	3, 4	(7) 8	Stevens, '09
Aphis oenotherae	4, 5	9 10	Stevens, '05, '06
Aphis ('golden-rod')	(5) (6)	(11) 12	Stevens, '09
Aphis ('rose aphis, green')	6, 7	(13) (14)	Stevens, '06, '09
Aphis (migratory)	(8) 9	(17) 18	Stevens, '06

Thus, in all these species, the number of sex chromosome is found to be only one in the male. So, Morgan, Sturtevant, Müller and Bridges ('15), Doncaster ('24), and others were right in stating that "there are only two sex-chromosomes in the aphid" (Morgan, etc., p. 103); and "In the aphids, however, as in other parthenogenetic forms, only females arise from fertilized eggs, and since the male has only one X-chromosome one would expect that two kinds of spermatozoa would be formed" (Doncaster, p. 165). Since we have found at least one species of aphidids with four sex chromosomes (in the male), such statements can no longer hold true, and consequently a modification in the wording becomes necessary.

So far as the present investigation goes, no case of what seems to be polyploidy was noticed. So, we shall at once consider other possibilities through which the increase or decrease in the number of chromosomes is brought about.

Two opposing views have been advanced by cytological workers in regard to the derivation of different numbers of chromosomes in animals and plants. One of these views regards the larger numbers of smaller chromosomes as the segmentation products of the smaller number of larger chromosomes, whereas the other considers a few larger chromosomes as resulting from the coalescence of small chromosomes. Thus, according to the former, the smallest number of larger chromosomes must be considered the most primitive, but just the opposite of this will become true in the case of the latter. Since, as we shall presently show, the increase in number of chromosomes is, for the most part, correlated with somatic complexity, we shall assume that the smallest number of chromosomes is the more primitive. The smallest known somatic number of chromosomes among animals is two, Ascaris megalocephalus var. univalens, studied by Boveri ('87). This number in Ascaris is now regarded by many authorities as representing a compound structure, but we shall take it as it is. Starting, then, from this primitive number, it became doubled in the next higher scale of evolu-

tion and thus produced animals with four (diploid number of) chromosomes. Evolution at this point must have proceeded in several directions, because the same number of chromosomes, four, is found in animals belonging to several phyla. It seems to have been at this stage that the class Insecta came to exist, for we have at least one insect with this number of chromosomes, and that insect is Icerya purchasi Maskell studied and reported by Pierantoni ('12) and figured also by me ('19). The fact that the least number of chromosomes found in the present-day aphids is five (diploid number in the male) suggests that the aphids are more modern insects than coccids, and that they probably originated when the diploid chromosomes found in Icerya-like insects became triploid, i.e., six in the female, and that the number five in the male cells was probably produced by the disintegration of the mate of one of the third, the XY pair. This is a very important point in the discussion of the evolution of the aphidids and their chromosomes.

The view just stated, that the aphids probably originated at the time when four chromosomes like the ones found in Icerya became six, and in consequence of this, that the forms possessing this number of chromosomes may be regarded as more primitive than those having a greater number of chromosomes, is in strict accordance with the views of Baker arrived at through anatomical and biological studies, for in part he says:

> When the phylogeny of this subfamily is studied, there becomes evident the primitive character retained by the Lachnini. In these forms the beak structure and the nature of the antennae and cornicles points to a primitive condition. . . . But the fact that these forms are mostly conifer feeders should not be overlooked. The Lachnini branch, therefore, may be considered the lowest branch of the Aphididae (Baker, '20, p. 4).

Before going into a still further discussion on the evolution of aphids, it will be of interest to review the data obtained through our cytological work. The following table summarizes the chromosomal numbers of the aphids so far investigated and reported in this treatise:

MATERIAL NO.	NAME OF SPECIES	TOTAL NUMBER OF ELEMENTS IN SPERMATOCYTE I	UNIVALENT ELEMENT IN SPERMATOCYTE I
	Tribe Lachnini:		
	Subtribe Lachnina:		
1	Dilachnus laricolus	5	1
2	Dilachnus pinidensiflorae	11	1
3	Eulachnus piniformosanus	7	1
	Subtribe Pterochlorina:		
4	Tuberolachnus viminalis	4	1
5	Pterochlorus tropicalis	8	?
	Tribe Callipterini:		
	Subtribe Phyllaphidina:		
6	Shivaphis celti	3	1
7	Phyllaphis fagi	13	1
	Subtribe Callipterina:		
8	Chromaphis magnoliae	4	1
9	Myzocallis castanae	7	1
10	Euceraphis betulae	6	4
11	Tuberculatus quercicola	7	1
12	Tuberculatus kashiwae	7	1
13	Callipterus kuricola	7	1
14	Therioaphis shinae	7	1
15	Calaphis betulaecolens	9	1
16	Calaphis magnolicolens	10	1
17	Symydobius kabae	13	1
	Subtribe Drepanosiphina:		
18	Drepanosiphum platanoides	15	1
19	Drepanaphis acerifoliae	19	1
	Subtribe Chaitophorina:		
20	Chaitophorus sali-apterus	7	1
21	Chaitophorus sali-niger	7	1
22	Periphyllus aceris	10	1
23	Periphyllus koelreuteriae	10	1
	Subtribe Pterocommina:		
24	Melanoxantherium sali-japonica	4	1
	Tribe Aphidini:		
	Subtribe Aphidina:		
25	Cavariella oenauthi	4	1
26	Carolinaia tade	5	1
27	Aphis sambuci	6	1
28	Acaudus itadori	6	1
	Subtribe Macrosiphina:		
29	Akkaia polygoni	6	1
30	Myzus suguri	6	1
31	Amphorophora ribicola	6	1
32	Amphorophora lespedezae	6	1
33	Amphorophora magnoliae	6	1
34	Macrosiphum gobonis	7	1
35	Macrosiphum sonchi	6	1
36	Macrosiphum ibotum	7	1
37	Macrosiphum cornifoliae	7	1

Now, let us again take up the question of evolution. As the table shows, the genera Tuberolachnus, Shivaphis, Aphis, and Carolinaia may be regarded as the ones originated from the trunk aphid with the least diploid number of chromosomes in

each of the main branches, but evolved toward a different direction.

So much for the origin and evolutional tendencies of the three main groups of aphids. Now let us go a little further into this question and investigate as much as possible the evolution of genera themselves. To begin with, the tribe Phyllaphidini, to which Shivaphis celti belongs, is considered by Baker ('20) to have branched out of the main aphid trunk after the Chaitophorina had differentiated from the Callipterini branch. At this point my interpretation is at variance with his. In my opinion the Chaitophorina branch with four or seven elements must have evolved much later than the Phyllaphidina with three elements, and the Chaitophorina with four elements must have originated just about the time when Melanoxantherium, with the same number of elements, had branched out from the Callipterini stock. In other words, both Chaitophorina and Callipterina might have simultaneously come from the same stock shortly after the Shivaphis had evolved.

Another difference in view regarding evolution, between morphologists on the one hand and cytologists on the other, pertains to the evolution of the subtribe Macrosiphina. Scanty as the materials are, I am convinced that the subtribe Macrosiphina has, in all probability, branched out of the Aphidina branch after such genera as Carolinaia and Aphis had been established as such, and that the time of separation must not, in all probability, have been earlier than the evolution of Acaudus. This assumption is, of course, based upon the chromosome numbers. The least diploid number of chromosomes found in the Macrosiphina group is twelve in the female. The known Aphidina having more than five (in the male) elements ($5_{II} + X$) is Acaudus. It is this fact that makes me believe that the Macrosiphina must have evolved a short time after, or simultaneous with, the appearance of the genus Acaudus.

One more point of disagreement, and that concerns the status of the subtribe Pterocommina. Baker ('20) considers this subtribe much higher than the subtribe Chaitophorina. But as we have found that the least number of elements in any

Chaitophorus so far investigated is seven, against four in the Pterocommina, we can but consider the latter much lower than the former. In fact, we think that the subtribe Pterocommina, to which our Melanoxantherium belongs, must have originated at about the time when Chromaphis with the same chromosome complex has separated from the subtribe Callipterina.

In all other points, the views of Baker, arrived at through anatomical and biological investigations, agree, for the most part, with my views arrived at through the cytological investigation of the germ cells. The accompanying figure (fig. A) illustrates the evolution of aphids according to the numbers and characters of their chromosomes.

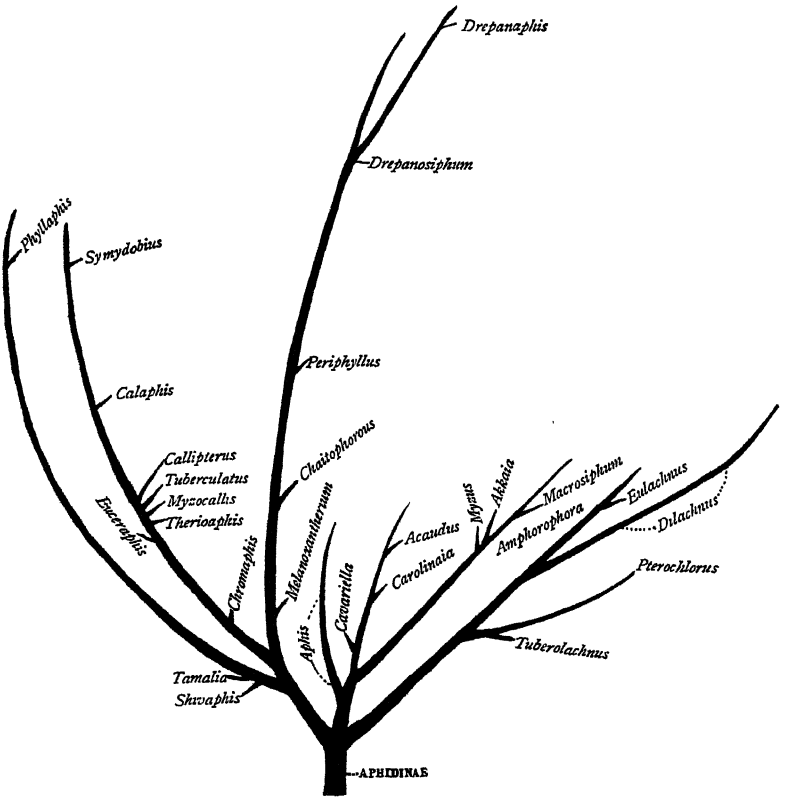
2. Evolution of the chromosomal numbers

Since, as stated above, the evolution of the genera and species of aphids is, for the most part, in accordance with the increase in number of chromosomes, and since the genera and species with the least number of chromosomes represent the most primitive ones morphologically, no objection will be raised against an assumption that the most primitive number of chromosomes is $2_{11} + X = 5$ (diploid in the male), and that all the other numbers are but the products of this primitive set. Taking this hypothesis as the basis of our discussion, we shall see how the different numbers of chromosomes found in our material can be accounted for.

To begin with, we have only one species that has four X chromosomes as against one in all other species so far studied. This species is Euceraphis betulae. Here the X elements, four in number, are individually and collectively smaller than any one of the bivalents in the spermatocytes. Thus, we may think that they are, in all probability, derived by the cross division of a single primitive X element. This assumption of the origin of the numerous X elements in aphids is just what Wilson ('09), for Syromastes marginatus, and Payne ('09), for many species of Reduviidae, have maintained. When, however, we examine the equatorial plate of the spermatogonia, the assumption becomes questionable, for

here each X element, except one, is nearly as large as any of the autosomes. Thus, I cannot help but surmise that at least two larger X elements of Euceraphis might have arisen when either a bivalent element or the mate of the X of the parthenogenetic form passed over into the same daughter cell to become two univalent elements.

In this connection it may be mentioned that our Euceraphis betulae is the first example of an animal with four X and no Y chromosomes in the male, although animals with two X components have been recorded in Lepisma (Charlton, '21), Syromastes (Gross, '04), Phylloxera (Morgan, '08), Agalena



A phylogenetic tree of APHIDINAE

Figure A

(Wallace, '05), Anolis (Painter, '21), and Scoloporus (Painter, '21). Again, animals with four or more X chromosomes and with or without Y element have been known, but, as stated, our Euceraphis betulae is the first example with four X's and no Y element in the male cell.

The next question that arises is: How have the autosomes of aphids multiplied? To this our answer is that they have, in all probability, multiplied by transverse division of primitive elements, so far as our material is concerned. Such cases of fragmentation of chromosomes is not altogether rare. The *m*-chromosomes, very minute chromosomes at present known to occur only in the coreid Hemiptera, where they were first described by Paulmier ('99) in Anasa tristis and since found in the other coreids, are examples of the fragmentation of the chromosomes resulting in the increase of the chromosome number. Another example, which, is, by the way, more convincing as to the origin of new chromosomes by cross division of larger ones, has been suggested by Kawaguchi ('23) in the case of Bombyx mori and its wild variety Bombyx mori var. mandarina. He states that there are twenty-seven chromosomes (haploid) in Bombyx mori var. mandarina, but this number is found increased to twenty-eight in Bombyx mori, and in crosses between these two forms, two of the twenty-eight chromosomes of the latter behave as though they were homologues of a single large one found in the former, showing their probable identity. Cases suggesting this kind of cross division of one or more of the chromosomes have also been clearly demonstrated in the pig and in Oenothera scintillans by Hance ('18). Similar cases have also been reported by Kuwada ('19) for Zea mays and by Carothers ('13, '17) and Robertson ('15) for certain grasshoppers. In all these cases the process of fragmentation did not alter the total chromatin mass. Thus the increase in number of chromosomes by their cross division is by no means rare, and I am strongly of the opinion that the different numbers of chromosomes observed in aphids, too, may have arisen by a similar process. Should this supposition turn out to be correct, then, it follows that the genera Aphis, Myzus, Acauda, and Amphorophora, each

of which with six chromosome elements, are the products of the elements of Carolinaia type when one of them underwent a cross division, and that Macrosiphum ibotum with seven chromosomes likewise might have been produced when one of the six elements of Macrosiphum sonchi underwent fragmentation. In this way all modifications in the number of chromosomes may satisfactorily be explained.

The existence of ten elements in Periphyllus koelreuteria and Periphyllus aceris is one of the interesting cases that suggest the probable segmentation of an element. The equatorial plates of these two species are almost similar, each with one tiny element besides eight middle-sized autosomes and one large X element. This small element in both cases is usually found near or closely attached to the larger one, suggesting that they have probably been produced by the fragmentation of one of the larger elements.

Another instructive example of similar nature is found in the case of Calaphis betulaecolens and Calaphis magnolicolens. In the former there are nine elements, including two very large rod-shaped elements, and in the latter there are one large and eight median ones besides a single small element which cannot be found in the former. Thus, although no direct evidence has yet been found, it is supposed that the smallest element has, in all probability, been produced by a cross division of one of the larger ones. Another example is found in Drepanosiphum platanoides and Drepanaphis acerifoliae. In the former there are fifteen elements, including four larger elements, whereas the latter shows nineteen elements of smaller type. So, we can but think that these four larger elements of the former have undergone a division to produce eight smaller ones.

The chromosome group found in Acaudus itadori is of interest because it can also be explained by the so-called non-disjunction hypothesis, for there are two small elements of apparently the same size and shape. But, since we have never observed a case in which two of the mates of a bivalent element pass to the same pole during meiosis, as Gates ('15) has found in Oenothera, we rather take this case, too, as

due to a cross division of one of the four bivalent elements found in such a species as Carolinaia tade.

Still another chromosome set that demands an especial consideration is that found in Dilachnus pinidensiflorae with eleven elements against five in its closely allied species, Dilachnus laricis. As some of its chromosomes are similar, we might take this number eleven as a case of reduplication like that of Oenothera gigas (Gates, '15). But since the total mass seems to be apparently the same as that of Dilachnus laricolus, we think it wise to regard this case, too, as one of a cross division of the chromosomes.

So much for the causes of varying numbers of chromosomes. Now, let us consider for a moment the probable cause of the production of chromosomes of varying sizes. The presence in each of the three main divisions or tribes of the subfamily Aphidinae of one or more genera or species with the same number and size of a chromosome complex, e.g., four, five, six, seven, ten, and thirteen elements, indicates that, in all probability, there may exist certain definite points of weakness, where a cross division may occur under certain conditions. That this is not altogether an unwarranted conclusion can be shown by the fact that in certain grasshoppers studied by Carothers ('17) and Robertson ('15) the break of the heteromorphic chromosome pairs takes place at a particular point marked by two large chromosome vesicles at which the spindle fibers are attached. Again, in some species, according to Wilson ('24) and others, the presence of cross sutures at certain points in the chromosomes can be demonstrated. Accordingly, we are of the opinion that all the different numbers of chromosomes found, at least in the same tribe, must have originated from those of the least in number, i.e., the fewer the chromosomes, the more primitive the species.

The hypothesis that the chromosomes of primitive species may have several points of segmentation leads us to another speculation that the species, or genera, having chromosomes of the same number, shape, and size may, in reality, be different, at least in the metabolic condition of the cells that contain them. To make this statement more concrete, let us imagine

that one of the two primitive autosome elements, AB and CD, of an aphid has segmented to become two at one of its points of fragmentation, S on AB; then the resulting combination AS + SB + CD + X (X being X chromosome) may manifest a different character from CS′ + S′D + AB + X, the resulting product of segmentation that has taken place in the other autosome element CD. With the increase in the number of points of fragmentation, numerous combinations of different elements or chromosomes differing in character, but of the same number, may be expected. The cases of Tuberolachnus, Cavariella, Chromaphis, and Melanoxantherium, belonging to four different subtribes having four chromosomes each, may be cited in favor of such a speculation. In like manner, we may devise the formulae to distinguish the chromosomal characters for each of the genera with the chromosome number of six, seven, nine, thirteen, and so on.

Although no conclusive evidence has been presented in favor of the view that the animal, the chromosome or chromosomes of which have become fragmented, shows different body characters from those individuals whose chromosomes have suffered no fragmentation, I can but think that in aphids, as in the case of Bombyx mori and its variety mandarina, the fragmentation of one or more of the pre-existing chromosomes may probably cause some body changes. On this point a discussion will be given in a separate paper to be published in the near future.

3. On the importance of the chromosome number for systematic studies

Stevens ('06) entertained an opinion that the system of classifying aphids at that time was unsatisfactory and that a new one based upon cytological investigations should be devised. My investigation into the numbers and sizes of nearly forty species representing some twenty-six genera has shown the fact that, in general, the present taxonomy of the family Aphididae as revised by Baker ('20) is for the most part in accord with the data obtained through cytological researches.

Since, as already stated, the number, the relative sizes and shapes, etc., of the chromosome elements are nearly constant in most of the species of the same genus, these chromosome characters may well be applied to the determination of doubtful genera. The following are some of the examples in which such doubtful genera may thus be given their proper positions:

a. The genera Tuberolachnus and Pterochlorus. The genus Tuberolachnus was erected in 1908 by Mordwilko with Lachnus viminalis Fonscolombe as type. This genus is distinguished from other genera of the tribe Lachnini by the possession, on the dorsum of its abdomen, of a very large tubercle, but Baker ('20) thinks that this character is insufficient for a distinction and, therefore, it ought to be placed in the same genus with Pterochlorus tropicalis. The result of my cytological work reported in this paper is to the effect that the sole representative of the genus Tuberolachnus in this country possesses four elements as against eight in Pterochlorus tropicalis. So, I am of the opinion that our viminalis should once more be placed under its old generic name of Tuberolachnus.

b. The genera Myzocallis, Callipterus, and Tuberculatus. Baker ('24) has recently reported the following list of synonyms for these three genera as follows:

A. Genus Myzocallis Passerini
- Myzocallis Passerini 1860
- Pterocallis Passerini 1860
- Subcallipterus Mordwilko 1894
- Tuberculatus Mordwilko 1894
- Callipterus v. d. Goot 1913
- Tuberculoides v. d. Goot 1915
- Acanthocallis Matsumura 1917
- Takecallis Matsumura 1917

B. Genus Callipterus Koch
- Callipterus Koch 1855
- Callaphis Walker 1870
- Ptychodes Buckton 1881
- Panaphis Kirkaldy 1904
- Nippocallis Matsumura 1917

The result of my cytological work is in strict accordance with the determination of Baker above mentioned. The species belonging to the genera listed and their chromosome numbers are as follows:

Name of species	*Number of chromosomes*
Myzocallis castanae	$6_{II} + X = 13$ (male, diploid)
Tuberculatus (Acanthocallis) quercicola	$6_{II} + X = 13$ (male, diploid)
Tuberculatus kashiwae	$6_{II} + X = 13$ (male, diploid)
Callipterus kuricola	$6_{II} + X = 13$ (male, diploid)

Thus, so far as the chromosome number alone is considered, there is no objection to placing these four species in the same genus. But Baker ('20) has proposed to separate the genus Callipterus from the rest. Cytological difference between the genera Tuberculatus and Callipterus is, as already mentioned, not so great as that existing between the genera Tuberculatus and Myzocallis proper. So, I wish to propose here that the genus Myzocallis of Baker should be divided into two genera of Myzocallis and Tuberculatus.

Key for separating the genera Myzocallis, Tuberculatus, and Callipterus

1. With a small *m*-element; antennae almost bare. . . . . Myzocallis
   With no very small *m*-element; antennae with long bristles 2
   tubercles which are paired and conspicuous. . . . Tuberculatus
2. With a large X chromosome; thorax and abdomen with dorsal tubercles which are paired and conspicuous Tuberculatus
   With a large X chromosome; thorax and abdomen without dorsal tubercles . . . . . Callipterus

c. The genera Euceraphis, Calaphis, Symydobius, and Yezocallis. The following tabulation brings out the differences and similarities existing among the species belonging to four supposedly different genera:

Name of species	*Number of elements*
Euceraphis betulae	$4_{II} + 4X = 8$ (♂)
Calaphis betulaecolens	$8_I + X = 9$ (♂)
Calaphis magnolicolens	$9_I + X = 10$ (♂)
Symydobius (Euceraphis) japonica	$12_I + X = 13$ (♂)
Symydobius (Yezocallis) kabae	$12_I + X = 13$ (♂)

The table clearly shows that Essig and Kuwana's Euceraphis is not a Euceraphis. Cytologically, again, at least no

difference can be detected between Euceraphis japonica and Yezocallis kabae which is, according to Baker, a Symydobius.

d. The genera Chaitophorus and Periphyllus. The chromosomal characters of the following four species formerly listed under the genus Chaitophorus will be made the basis of the present discussion:

Name of species	*Number of elements*
Periphyllus (Chaitophorinella) koelreuteriae	10 (♂)
Periphyllus (Chaitophorinella) aceris	10 (♂)
Chaitophorus sali-niger	7 (♂)
Chaitophorus sali-apterous	7 (♂)

The investigation into the chromosome number of these species has clearly brought out the existence of two well-defined groups, one possessing $9_{II} + X = 10$ elements, and the other, $6_{II} + X = 7$. So my views of the classification of these genera are in strict accordance with those of Baker ('20).

SUMMARY

1. There is but one sex chromosome in the male cells of all but one species of Aphididae. That exceptional species is Euceraphis betulae Koch, which has four X chromosomes.

2. The sex chromosome or chromosomes become drawn out during the anaphase to form the 'lagging chromosome' of Stevens. Finally, however, they are all withdrawn in their entirety into one of the two resulting second spermatocytes. As a result of this peculiar behavior of the sex chromosomes, two kinds of the second spermatocytes are formed: one with, and the other without, the sex chromosome. The latter kind degenerates in situ, while the former produces the functional spermatozoa after another division.

3. The number of chromosomes and specific body characters are so closely correlated that we can safely judge the evolutional scale of any aphid by its number of chromosomes.

4. So far as the present investigation goes, it seems that in the family Aphididae the least number of chromosomes is the most primitive, and that the numerous other numbers have all evolved from that primitive number.

5. The numbers of chromosomes found in the subfamily Aphidinae vary, for the most part, with the genera. The

highest number is $18_{II} + X = 37$ chromosomes, the lowest being $2_{II} + X = 5$ (diploid in the male).

6. The increase in number of chromosomes seems to have been made by a transverse division or divisions of one or more of the primitive chromosomes.

7. The number and the relative sizes and shapes of the chromosomes are, for the most part, constant within a genus, and so these characters may generally be used in the determination of genera.

8. The genus Tuberolachnus with $3_{II} + X = 4$ (♂) elements should be separated from the genus Pterochlorus with eight (♂) elements, and called again by its former generic name of Tuberolachnus.

9. In the genera Periphyllus and Calaphis there are species with a small *m*-element which is bivalent.

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PLATES

The drawings were made with the aid of a camera lucida. All drawings were originally magnified 3040 times, using a 20× ocular and 1/12 objective, N. A. 1.3. They were afterward reduced to about 2280 in reproduction.

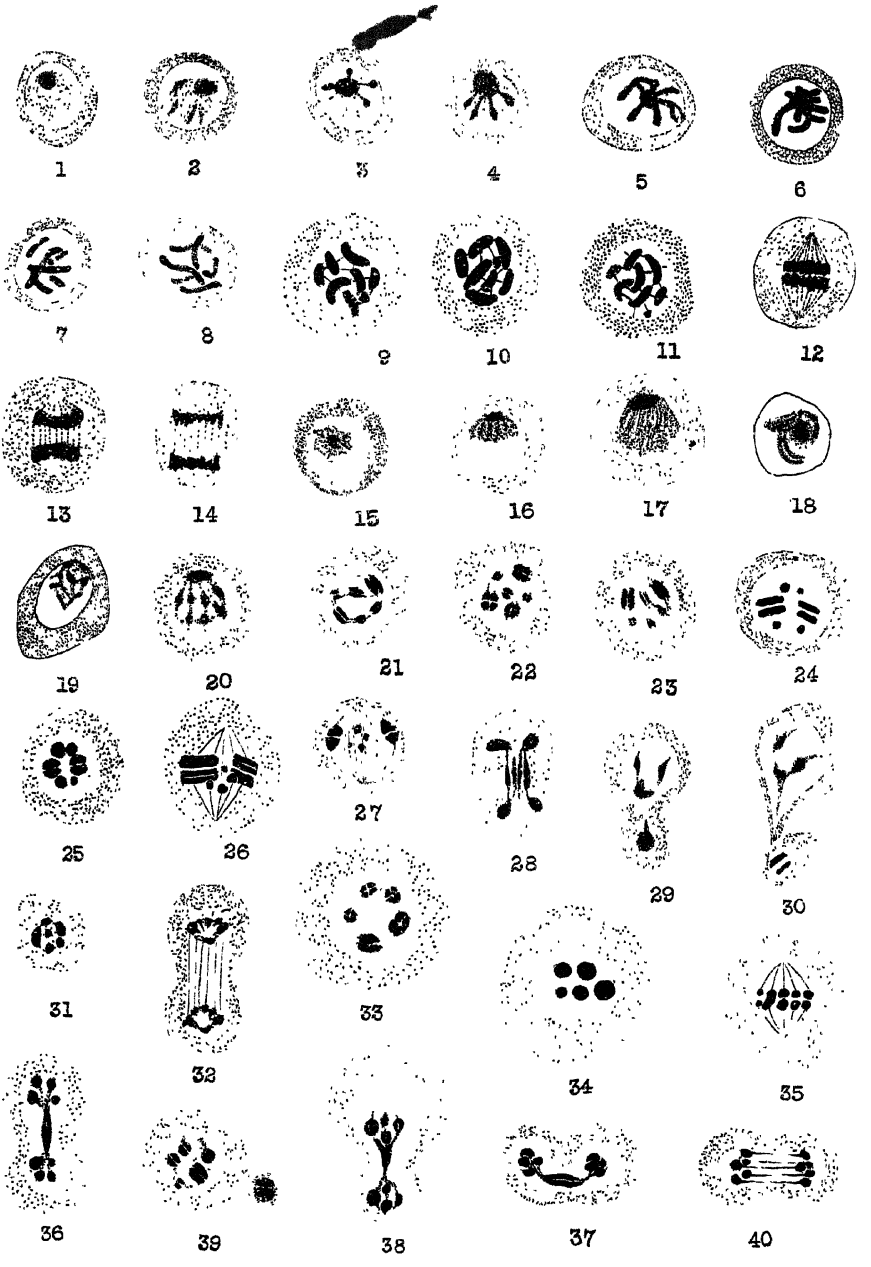
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PLATE 1

EXPLANATION OF FIGURES

Spermatogonia of Euceraphis betulae (8 chromosomes)

1 Resting spermatogonium.
2 A little later stage.
3 and 4 Still more later stage. Chromosomes began to appear.
5 and 6 Prophase. The chromosomes have already assumed the shape recognizable as such.
7 and 8 All the chromosomes have already emerged, and in figure 7 they are forming a thread, almost continuous.
9 to 11 Equatorial plates of the metaphase.
12 Lateral view of the equatorial plate.
13 and 14 Anaphase. Chromosome plates are still seen advanced to the opposite poles.

Spermatocyte I of Euceraphis betulae (8 chromosomes)

15 to 22 Various stages in the growth period.
23 and 24 Prophase figures showing parasynapsis.
25 Metaphase, a polar view.
26 and 27 Metaphase, lateral views.
28 Anaphase, showing the lagging chromosomes.
29 and 30 Telophase, showing two kinds of daughter nuclei.

Spermatocytes II of Euceraphis betulae ($2_{II} + 4X = 6$ elements)

31 Metaphase, a polar view.
32 Anaphase, a lateral view.

Dilachnus laricis ($4_{II} + X = 5$ elements)

33 A spermatocyte of the first order in late prophase.
34 Polar view of the metaphase of the first maturation division.
35 Lateral view of the metaphase of the first maturation division.
36 to 38 Lateral views of the spermatocyte of the first order in anaphase.
39 Prophase of the second maturation division. A small cell near by represents a degenerating spermatocyte II.
40 Anaphase of the second maturation division.

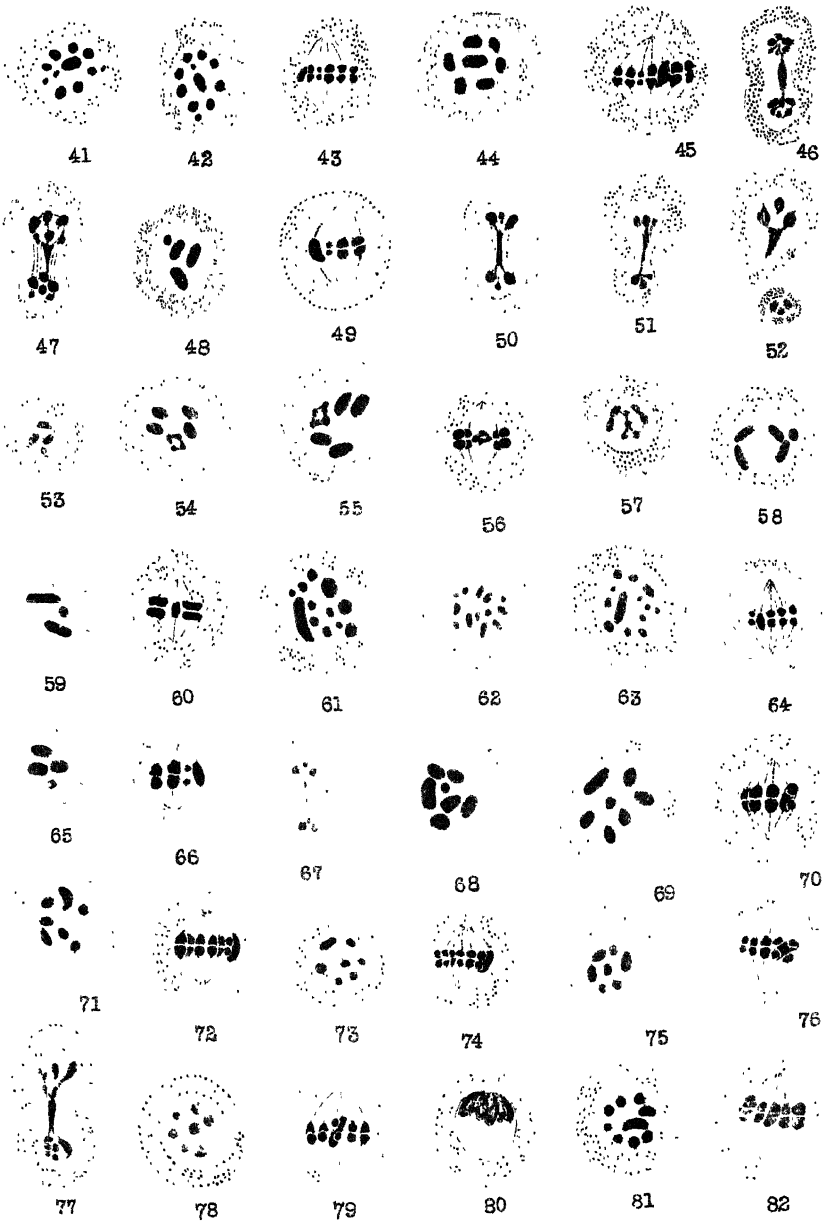
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PLATE 2

EXPLANATION OF FIGURES

Dilachnus pinidensiflorae ($10_{II} + X = 11$ elements)

41 and 42 Polar views of the equatorial plate of the first spermatocyte division.
43 Lateral view of the equatorial plate of the first maturation division.

Eulachnus piniformosanus ($6_{II} + X = 7$ elements)

44 Semipolar view of the equatorial plate of the first maturation division.
45 Lateral view of the equatorial plate of the first maturation division.
46 and 47 Anaphase of the first maturation division.

Tuberolachnus viminalis ($3_{II} + X = 4$ elements)

48 Polar view of the metaphase plate of the first maturation division.
49 Lateral view of the equatorial plate of the first maturation division.
50 to 52 Lateral views of the anaphase of the first spermatocyte.
53 Polar view of the equatorial plate of the second spermatocyte.

Pterochlorus tropicalis ($4_{II}? + ?X = 8$ elements)

54 and 55 Polar views of the equatorial plate of the first spermatocyte.
56 Lateral view of the metaphase plate of the first spermatocyte. Photomicrograph of the same figure is no. 168, plate 5.
57 The second spermatocyte in metaphase.

Shivaphis celti ($2_{II} + X = 3$ elements)

58 Prophase of the first spermatocyte division.
59 Polar view of the equatorial plate of the first maturation division.
60 Lateral view of the equatorial plate of the spermatocyte I.

Phyllaphis fagi ($12_{II} + X = 13$ elements)

61 to 63 Polar views of the equatorial plate of the first maturation division.
64 Lateral view of the equatorial plate of the first maturation division.

Chromaphis magnoliae ($3_{II} + X = 4$ elements)

65 Polar view of the equatorial plate of the first maturation division.
66 Lateral view of the equatorial plate of the first maturation division.
67 Anaphase of the first maturation division.

Myzocallis castanae ($6_{II} + X = 7$ elements)

68 and 69 Polar views of the equatorial plate of the first maturation division.
70 Lateral view of the equatorial plate of the first maturation division.

Tuberculatus quercicola ($6_{II} + X = 7$ elements)

71 Polar view of the equatorial plate of the spermatocyte I.
72 Lateral view of a similar plate.

Tuberculatus kashiwae ($6_{II} + X = 7$ elements)

73 Polar view of the equatorial plate of the first maturation division.
74 Lateral view of a similar plate.

Callipterus kuricola ($6_{II} + X = 7$ elements)

75 Polar view of the equatorial plate of the first reduction division.
76 Lateral view of a similar figure.
77 Anaphase figure of the first maturation division.

Therioaphis shinae ($6_{II} + X = 7$ elements)

78 Polar view of the equatorial plate of the first maturation division.
79 Lateral view of the equatorial plate of the first maturation division.

Calaphis betulaecolens ($8_{II} + X = 9$ elements)

80 Contraction stage of the spermatocyte I.
81 Polar view of the equatorial plate of the first maturation division. This is the drawing of the same cell as the photomicrograph no. 177 represents.
82 Lateral view of the equatorial plate of the first maturation division.

PLATE 3

EXPLANATION OF FIGURES

Calaphis magnolicolens ($9_{II} + X = 10$ elements)

83 Prophase of the first maturation division.
84 and 85 Polar views of the equatorial plate of the first maturation division.
86 Lateral view of the equatorial plate of the spermatocyte of the first order.

Symydobius kabae ($12_{II} + X = 13$ elements)

87 and 88 Polar views of equatorial plates of the first maturation division. Figure 88 is photomicrographed as no. 170, plate 5.
89 Lateral view of the equatorial plate of the first spermatocyte.

Drepanosiphum platanoides ($14_{II} + X = 15$ elements)

90 Polar view of the metaphase, spermatocyte I.
91 Lateral view of the metaphase plate of the spermatocyte I.

Drepanaphis acerifoliae ($18_{II} + X = 19$ elements)

92 Prophase, polar view, spermatocyte I.
93 Polar view of the first maturation (reduction) division.
94 Lateral view of similar spermatocyte.
95 Late anaphase of the first spermatocyte division.

Chaitophorus sali-apterus ($6_{II} + X = 7$ elements)

96 Polar view of the metaphase of the first maturation division.
97 Lateral view of the equatorial plate of the first maturation division.

Chaitophorus sali-niger ($6_{II} + X = 7$ elements)

98 and 99 Polar views of the equatorial plates of the first spermatocyte.
100 A lateral view of the equatorial plate of the first maturation division.

Periphyllus aceris ($9_{II} + X = 10$ elements)

101 Polar view of the equatorial plate of the first maturation division. This figure represents the same cell as photomicrograph no. 180.
102 Lateral view of the equatorial plate of the spermatocyte of the first order.
103 Polar view of the equatorial plate of the second maturation division.

Periphyllus koelreuteriae ($9_{II} + X = 10$ elements)

104 Polar view of the equatorial plate of the first maturation division.
105 Lateral view of a similar spermatocyte of the first order.

Melanoxantherium sali-japonica ($3_{II} + X = 4$ elements)

106 Prophase of the first spermatocyte.
107 and 108 Polar views of the equatorial plate of the first maturation division.
109 Lateral view of the spermatocyte of the first order in metaphase.
110 Anaphase figure of the first maturation division.
111 Anaphase of the second maturation division.

Cavariella oenauthi ($3_{II} + X = 4$ elements)

112 Prophase of the first spermatocyte, showing one univalent and three bivalent elements.
113 Polar view of the equatorial plate of the first maturation division.
114 Lateral view of the equatorial plate of the first maturation division.
115 Anaphase of the first spermatocyte.
116 Polar view of the equatorial plate of the second maturation division.
117 Anaphase of the second spermatocyte.

Carolinaia tade ($4_{II} + X = 5$ elements)

118 Spermatocyte of the first order in a late prophase.
119 Polar view of the equatorial plate of the spermatocyte of the first order.
120 Lateral view of the equatorial plate of the spermatocyte of the first order.
121 and 122 Anaphase of the first (heterotypic) maturation division of the spermatocyte I.
123 Spermatocyte II in metaphase.

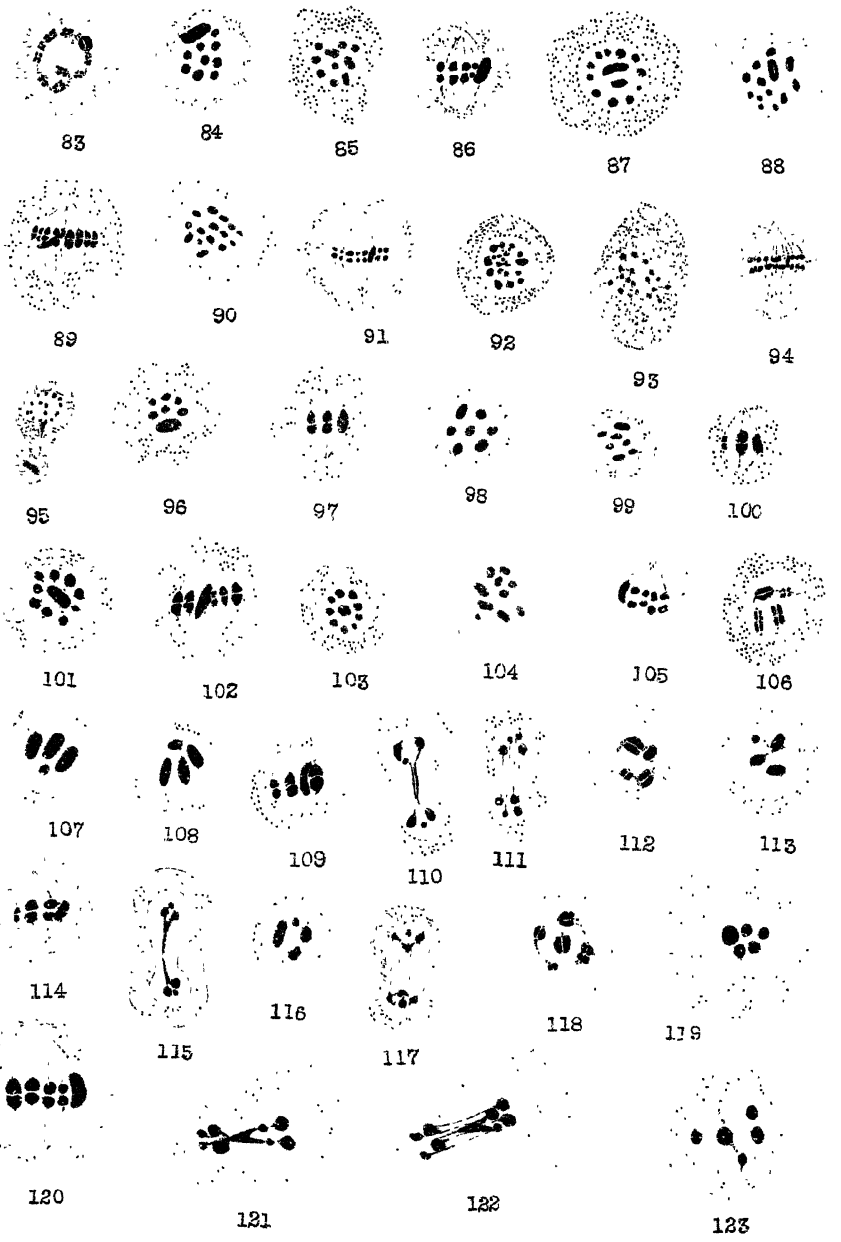
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PLATE 4

EXPLANATION OF FIGURES

Carolinaia tade (continued)

124 Anaphase of the spermatocyte of the second order.

Aphis sambuci (5II + X = 6 elements)

125 Polar view of the equatorial plate of the first maturation division.
126 Lateral view of the equatorial plate of the first maturation division.

Acaudus itadori (5II + X = 6 elements)

127 Polar view of the first maturation division. This will serve to show the relative size, shape, and position of the elements. This is the drawing of the same cell as photomicrograph no. 186.
128 A lateral view of an anaphase of the first maturation division.

Akkaia polygoni (5II + X = 6 elements)

129 Prophase of the first maturation division.
130 Polar view of the metaphase of the first maturation division.
131 Lateral view of the equatorial plate of the first maturation division.

Myzus suguri (5II + X = 6 elements)

132 Polar view of the equatorial plate of the first spermatocyte.
133 Lateral view of the equatorial plate of the first spermatocyte.

Amphorophora ribicola (5II + X = 6 elements)

134 and 135 Growth period, spermatocyte I.
136 Prophase, spermatocyte I.
137 Polar view of the first maturation division.
138 Lateral view of the same.
139 to 142 Lateral views of the anaphase of the first maturation division.
143 Polar view of the equatorial plate of the second spermatocyte mitosis.
144 Anaphase of the second spermatocyte division.
145 Semipolar view of the germinal vesicle or the oocyte I in metaphase.

Amphorophora lespedezae (5II + X = 6 elements)

146 Polar view of the equatorial plate of the first spermatocyte mitosis.
147 Lateral view of the equatorial plate of the first spermatocyte mitosis.
148 Lateral view of the anaphase plate of the first maturation division.
149 Polar view of the equatorial plate of the second spermatocyte mitosis.

Amphorophora magnoliae (5II + X = 6 elements)

150 Polar view of the equatorial plate of the first maturation division.
151 Lateral view of the equatorial plate of the first maturation division.

Macrosiphum gobonis (6II + X = 7 elements)

152 Polar view of the equatorial plate of the first spermatocyte mitosis.
153 Lateral view of the equatorial plate of the first spermatocyte mitosis.

Macrosiphum sonchi (5II + X = 6 elements)

154 Polar view of the equatorial plate of the first maturation division. This is the drawing of one of the cells shown in photomicrograph no. 188.
155 Lateral view of the equatorial plate of the first spermatocyte.
156 Lateral view of the anaphase figure of the first spermatocyte mitosis.

Macrosiphum ibotum (6II + X = 7 elements)

157 A polar view of the equatorial plate of the first maturation division.
158 Lateral view of the equatorial plate of the first maturation division.

Macrosiphum cornifoliae (6II + X = 7 elements)

159 and 160 Semipolar views of the equatorial plate of the spermatocyte of the first order.

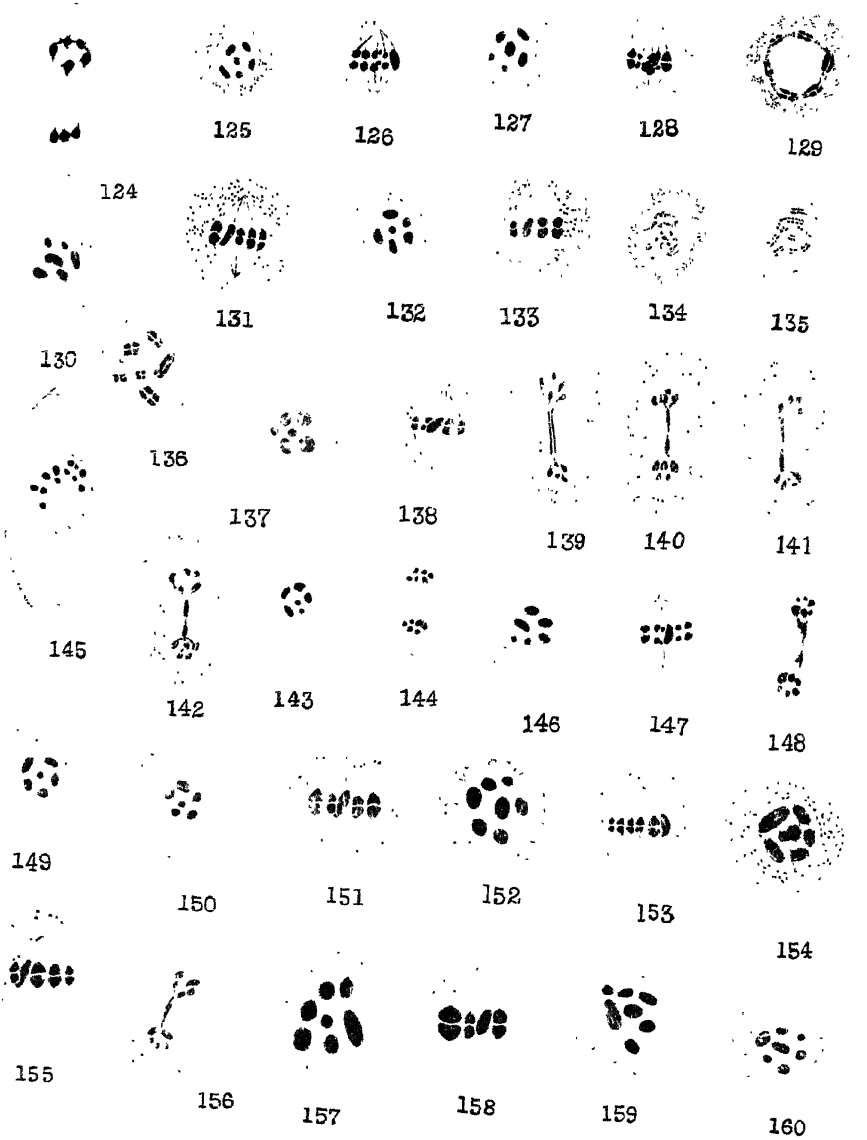
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PLATE 5

EXPLANATION OF FIGURES

(Photomicrographs of the first spermatocyte.)

161 Dilachnus laricolus. Polar view of metaphase plate.
162 Dilachnus pinidensiflorae. Polar view of metaphase plate.
163 Eulachnus piniformosanus. Polar view of metaphase plate.
164 Tuberolachnus viminalis. Polar view of metaphase plate.
165 Tuberolachnus viminalis. Lateral view of metaphase plate.
166 and 167 Pterochlorus tropicalis. Polar view of metaphase plate.
168 Pterochlorus tropicalis. Lateral view of metaphase plate. The same figure is also represented by figure 56.
169 Shivaphis celti. Polar view of metaphase plate.
170 Phyllaphis fagi. Polar view of metaphase plate. This is the same as figure 88. This photograph shows twelve out of thirteen elements, the remaining one being out of focus.
171 and 172 Chromaphis magnoliae. Polar view of metaphase plate.
173 Euceraphis betulae. Polar view of metaphase plate.
174 Lateral view of anaphase. Euceraphis betulae.
175 Callipterus kuricola. Polar view of metaphase plate.
176 and 177 Calaphis betulaecolens. Polar views of metaphase plate. This is the same figure as figure 81 represents.
178 Drepanosiphum platanoides. Polar view of metaphase plate.
179 Cavariella oenauthi. Polar view of metaphase plate.
180 Periphyllus aceris. Polar view of metaphase plate. The same figure is also represented by figure 101.
181 Periphyllus koelreuteriae. Polar view of metaphase plate.
182 Carolinaia tade. Polar view of metaphase plate.
183 Carolinaia tade. Lateral view of anaphase (left) and lateral view of metaphase (right).
184 Carolinaia tade. Lateral view of anaphase, showing lagging chromosome.
185 Aphis sambuci. Polar view of metaphase plate.
186 Acaudus itadori. Polar view of metaphase plate. This is a photograph of the cell much like the one represented by figure 127.
187 Amphorophora magnoliae. Polar view of metaphase plate.
188 Macrosiphum sonchi. Lateral view (left) and polar view (right) of metaphase plate. The same figure is represented by figure 154.
189 Macrosiphum cornifoliae. Polar view of metaphase plate.

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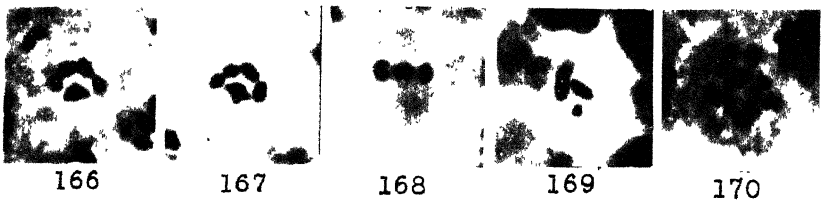
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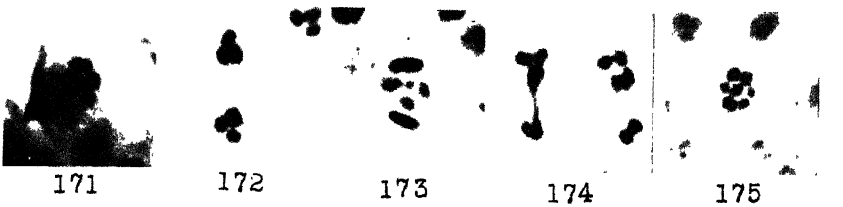
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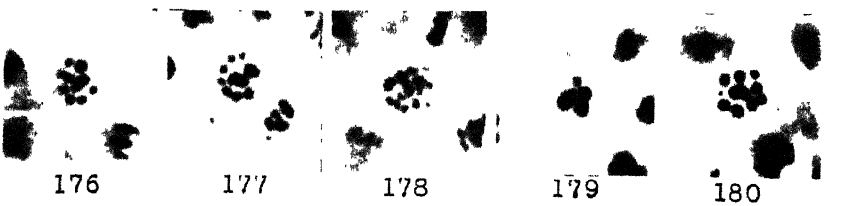
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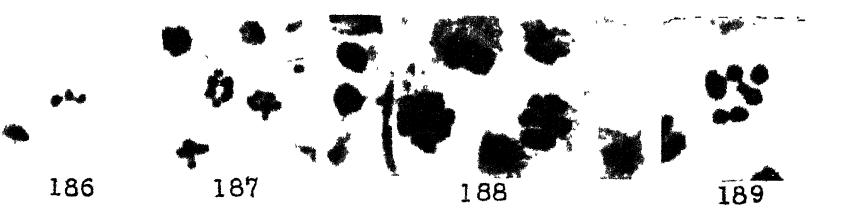
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RECOVERY FROM IMMERSION IN WATER. AN INDEX OF METABOLISM AND THE CONDITION OF THE GONADS IN DROSOPHILA MELANOGASTER MEIGEN AND POPILLIA JAPONICA NEWM.

MILTON WALKER EDDY

Zoölogical Laboratory, University of Pennsylvania

TWELVE FIGURES

AUTHOR'S ABSTRACT

In some insects the selection of individuals which recover in equal periods of time from immersion in water may be used to obtain material of approximately uniform physiological state; Drosophila melanogaster, Meigen, was found to have a mean of recovery for the males 0.97 ± 0.542 minute later than the females, which would indicate a lower metabolic rate for the males. The Japanese beetle, Popillia japonica Newm., was found to have a mean of recovery from immersion in water of both sexes the same in August, while in September the males were retarded by 8.1 as compared with females. This would indicate that the males have an equal metabolic rate in August with the females and a lower rate in September. Carbon dioxide, hydrogen, and nitrogen produce in the flies a condition of asphyxiation similar to immersion in water. 'Vestigial' flies recover from immersion in water in a fashion similar to 'wild' flies.

The recovery periods from immersion in water in the flies can be used to group individuals into those possessing approximately equal egg-laying capacities and to isolate spent individuals from others still capable of laying eggs. Yeast appears to be more of a stimulant to egg laying than it is to larval growth. Groups of flies which were fed yeast were found to average 71 per cent increase in egg laying over groups of flies that were not fed yeast. The O_2 consumption of the groups of flies actively laying eggs, as contrasted with the groups which were not so active, was found in the former to be 32.9 per cent greater.

INTRODUCTION

Physiological studies on the respiration rate of various groups of insects have usually employed the chronological age, or the weight of the individual, or both as a starting-point in experiments. The work of Morgan ('19) and his students has been largely concerned with genetical studies and has frequently included chronological age. In the physiological comparisons on nutrition which have been made by Loeb and Northrup ('16), Guyénot ('07), Baumberger ('19), Bonnier ('26), and Adolph ('20), the starting-point has been age. Pearl ('21 to '26) and his associates have been concerned with the duration of life along with the nutritional studies. The physiological differences between stocks and

strains of Drosophila, employing both age and weight, have been investigated in the pupal condition by Bodine and Orr ('25), Orr ('25), and Clare ('25). In the last work the author has pointed out that the weight of respiratory tissue alone is not a true index of metabolism in Drosophila and has suggested environmental influences. Some physiological studies on other groups of insects in which age and weight have been used are Bodine ('21) on grasshopper, Fink ('25) on potato beetle and other insects, Krogh ('14–'15) on Tenebrio and numerous other insects, and Taylor ('27) on blowflies, flesh flies, Mediterranean flour moth, and bee moth.

It frequently happens that individuals of the same age and weight do not fall within the expected respiration rate, as pointed out by Clare ('25) and others. For that reason there is need for employing some other method of dividing animals into groups of equal metabolic rate which would not necessarily employ age and weight. The following experiments indicate that individual insects, recovering from immersion in water or asphyxiation in equal periods of time, may be used as a means of dividing certain insects into groups of more nearly equal metabolic rates as evidenced by egg-laying capacity and O_2 consumption. The insects which recover from immersion in water or asphyxiation in each five-minute interval of time (immersion-in-water time and temperature being kept constant) were considered as unit groups and the plotting of the frequency curves of recovery was used in comparing various chronological age groups of 'wild' and 'vestigial' flies to determine whether age would cause a shifting of the mean. The effect of carbon dioxide, hydrogen, and nitrogen as agencies for asphyxiation was next considered. In all the experiments the question of the influence of sex upon recovery was investigated. Further experimentation dealt with the correlation of egg laying subsequent to immersion in water over periods of ten days; the effect of immersion in water on the fertility of eggs and the development of the larvae to pupa and adult; the rôle of yeast as a factor in larval development, and also as a gonadal stimulant for the

adult female; and finally, the increase in O_2 consumption of flies active in egg laying as compared with similar flies which were not laying as many eggs.

PROCEDURE

Drosophila melanogaster Meigen, in numbers of from 100 to 300, were placed in a cylindrical glass tube, 22 mm. in diameter and 150 mm. in length, both openings being closed by a single layer of cheese cloth held on by rubber bands. This tube was immersed in distilled water to a depth of 300 mm. for a period of fifteen minutes, the temperature of water being 25°C. ± 0.5°. At intervals of two minutes, during the period of immersion, the tube was rapidly shaken up and down, to separate the flies and to avoid an accumulation of air bubbles in which the flies would otherwise remain. After immersion in water, the flies were removed to moist paper toweling and spread out so that they were not in contact with each other. Air temperature was kept at 25°C. ± 1° during all of the experiments, and the temperature of the pad on account of evaporation varied from 21° to 23°C., due to the differences of humidity in the room. As recovery occurred the flies were transferred for future sex determination to Syracuse watch-glasses filled with 50 per cent alcohol, since recovery was so rapid occasionally that it was impossible to determine the sex of all flies immediately. The egg-laying cylinders were glass rings about 18-mm. inside diameter, 9 mm. in height, and cemented to 3 × 1 microscopic slides with de Khotinsky cement. These small egg trays were half filled with culture medium and were easily handled under a binocular microscope. As the culture medium was placed in each sterile tray it was covered with a larger ring on to which a circular cover-glass had been cemented. Considerable difficulty from the growth of mold and bacteria was encountered at first, since the cement used in the construction of the egg-laying trays could not be sterilized at high temperatures. The following procedure finally gave satisfactory results.

The trays were washed with soap and water and allowed to soak for at least six hours in a solution of lysol, then rinsed with distilled water and placed in 70 per cent alcohol for six hours, dried on paper toweling, and finally, just before filling with culture medium, were flamed with a Bunsen burner. At the beginning of each five-minute interval of recovery, five females that had recovered from immersion in water, instead of being placed in alcohol, were introduced into these egg-laying trays to determine the rate of egg laying during the next ten days. As the flies were introduced into these trays, their covers were removed and glass cylinders, 40 mm. in height and 24 mm. in diameter, with fine bronze-wire mesh tops cemented to them, were placed over the trays. The first five females to recover after immersion in water, during each five-minute interval, were placed in breeding cages and allowed to lay eggs for twenty-four hours, when they were transferred to fresh medium trays. Since Drosophila is strongly heliotropic (Carpenter, '05), especially when an unusually strong illumination is near, the flies would congregate near the wire gauze. Occasionally a fly would remain near the bottom and a light tap on the slide would cause it to fly to the top. This made possible the daily transference of the cylinder tops with the flies to fresh culture dishes, half filled with medium, and the daily counting of the number of eggs in the old dishes. During the egg-laying period the laying chambers were kept in incubators held at 25°C. $\pm$ 0.2°. Moreover, the gauze tops were covered with moist paper toweling to keep the humidity near the saturation point, since low humidity was found to retard the rate of egg laying.

Synthetic food medium was used during the first few experiments, as recommended by Pearl ('26), but the use of this did not result in high egg-laying rate. Guyénot ('13) noted that flies on poor nutrition laid fewer eggs and a modification of the medium originating from Morgan's laboratory was used.

H_2O,	500 cc.
Agar-agar,	10 grams
Banana,	500 cc.

The banana was passed through a ricer and added to the hot water, in which the "Bacto" agar-agar had been dissolved, then boiled for five minutes, and pressed through cheese cloth. The resultant medium, while it was not as transparent as the synthetic medium or as Baumberger banana medium ('19), gave satisfactory results.

During the course of these experiments fresh medium was prepared daily, except in a few cases and then every other day. In comparing the results no appreciable difference could be noticed in rate of egg laying when the medium was fresh or one day old.

EXPERIMENTAL DATA

In the analysis of data of recovery from immersion in water of 7389 flies they fall into three groups; first, cultures in which the flies are three days old and these are the first to emerge; secondly, cultures in which the flies are five or six days old; and, thirdly, old cultures in which no larvae are present and in which many of the flies are noticeably smaller than normal. The groups of flies three days old which are the first to emerge in a culture recover from immersion in water in five-minute intervals with such frequency as to produce a typical frequency curve as shown in figure 1. The mean of recovery for the males of all flies in this group was 13.3 minutes and for the females 13.8 minutes, with a probable error of males $\pm$ 0.3108 and females $\pm$ 0.2934; and the means of four groups of curves in this class for the males were 14.72 minutes and for the females 14.45 minutes, with probable errors of $\pm$ 0.398 and $\pm$ 0.3586, respectively. Daily egg laying is illustrated by the dotted lines on the graph, in number of eggs per female, and the egg laying over a period of ten days is shown to remain in an increasing series from the ten-minute period to the twenty-minute period. There is one marked exception in the case of the females in the ten-

minute recovery period on the tenth day, when a large increase in the number of eggs was secured which could be accounted for in two ways, either by an accidental introduction of a fly from the outside or by contamination of the medium by yeast.

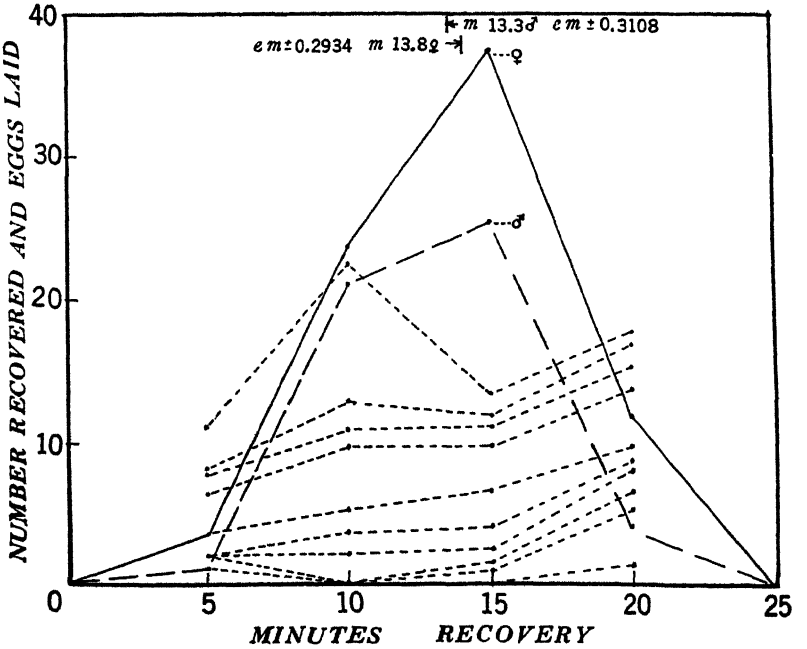

Fig. 1 Frequency curve of recovery from immersion in water (solid line represents females; broken line, males), and egg laying per day per female (shown by dotted lines) during ten days. These Drosophila were three days old. *m*, mean; *em*, probable error. Egg laying of the first type is shown in this graph, in which females recovering early lay more eggs than those recovering late. Each daily egg-laying curve was superimposed upon the previous day's egg-laying curve.

The second type of curve (figs. 2 and 3) was secured from flies five to six days old, which include many females at the height of their egg-laying capacity. An example of these curves is illustrated in figure 2, which includes flies six days old, where the mean of recovery is 22.1 minutes for the males and 18.9 minutes for the females, with probable errors of

$\pm$ 0.2846 and $\pm$ 0.2449. This curve was included in this group for two reasons; first, it shows the greatest difference secured between male and female means to be 3.2 minutes, and, secondly, the egg laying of the first ten-minute recovery group did not give the expected high rate which was usually secured from the first group to recover. Figure 3 gives

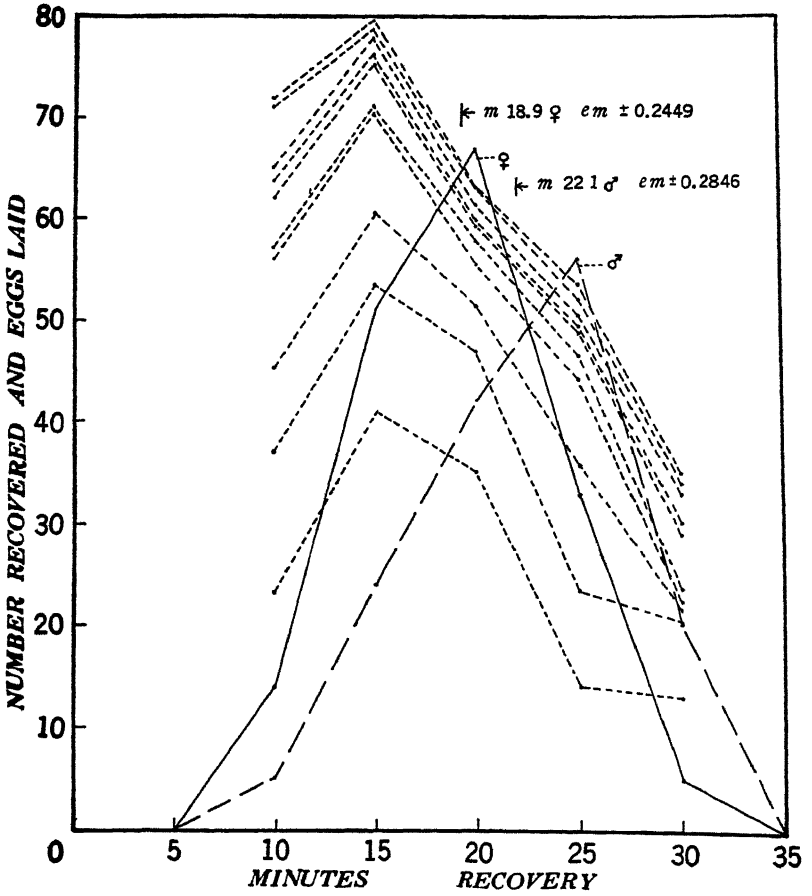

Fig. 2 Egg-laying curve for a period of ten days (shown by dotted lines) of the second type is illustrated in this graph, in which the females recovering early lay more eggs than those recovering late. Frequency curve of recovery from immersion in water is shown by solid line representing females and broken lines, males. These Drosophila were six days old. *m*, mean; *em*, probable error.

the best example of flies at the height of egg-laying rate correlated with earlier recovery and is the extreme in the direction toward younger flies. The mean for the male is 16.0 minutes, with a probable error of $\pm$ 0.2788, and for the

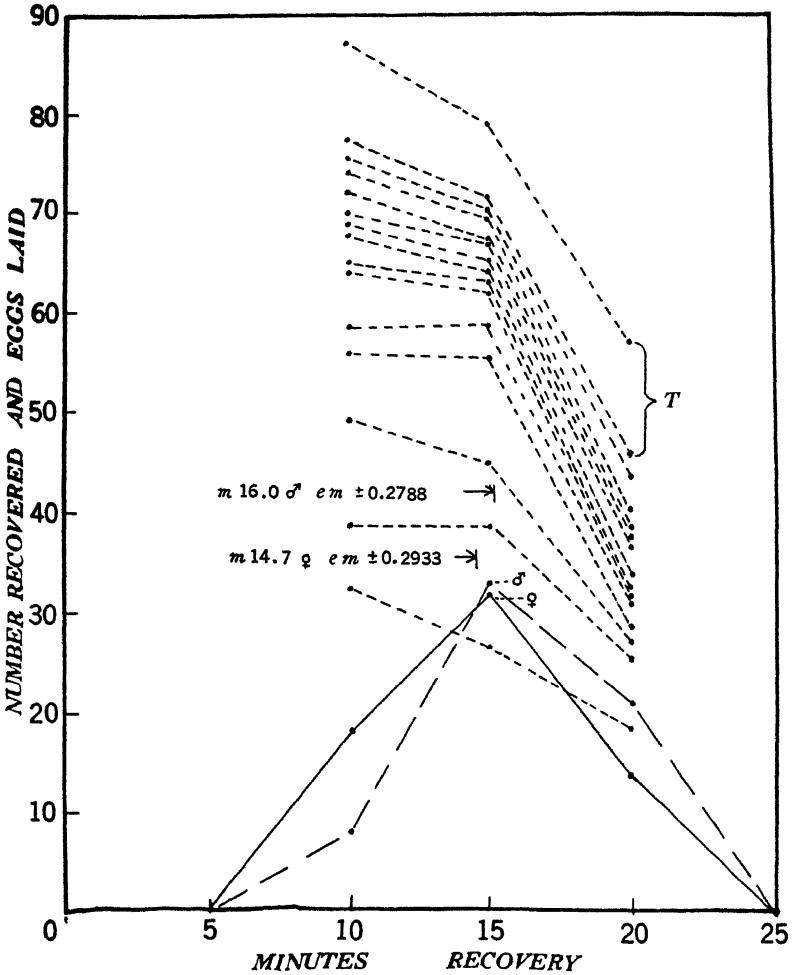

Fig. 3 The egg-laying curve for fifteen days in this graph (represented by dotted lines) is of the second type, in which the females recovering early lay more eggs than those recovering late, while the recovery periods from immersion in water are shown by solid line for females and broken line for males. *T*, temperature increase from 25°C. to 30°C., thereby increasing the rate of egg laying. These Drosophila were six days old. *m*, mean; *em*, probable error.

females the mean is 14.7 minutes with $\pm$ 0.2933 probable error. All the curves falling in this class gave a mean of 19.35 minutes for the males and 17.32 minutes for the females, with probable errors of $\pm$ 0.3025 and $\pm$ 0.2922. The number

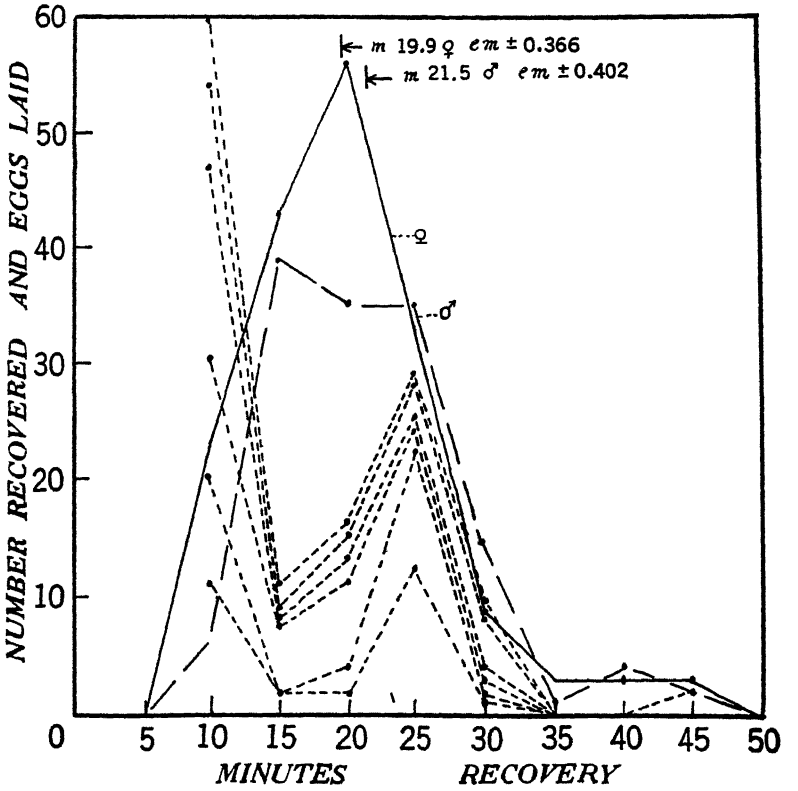

Fig. 4 Third type of recovery and with erratic egg laying during six days as secured from sixty-three-day-old culture of Drosophila; males, broken line; females, solid line; eggs per day per female are represented by dotted line.

of eggs laid by this group is greatest for the flies which recover within ten to twenty minutes and falls off to lower numbers in the last of the five-minute periods.

The third type of curve is shown in figure 4, which was obtained from very old cultures in which the worked-over food no longer contained larvae, and in these cultures a

large number of the flies are smaller in size. However, a number of the flies from this type of culture never recover from immersion in water and are therefore not included in the graph. In figure 4 the mean recovery time of the males is 21.5 minutes and that of the females is 19.9 minutes, with probable errors of $\pm$ 0.402 and $\pm$ 0.366. In this case many of the flies recovered at a very much later time than the two preceding examples, and while the means are very nearly the same as for the second type of curve, the egg laying was different in that many of the groups of five flies which recovered after thirty minutes did not lay any eggs during the ten days they were under observation and therefore were spent flies. Furthermore, nearly all the flies which recovered after thirty minutes died within a few days, and in most cases did not live longer than one day. During this period the egg-laying curve had a tendency to make marked departure from the expected curve, with a few of the egg-laying periods being erratic. This culture was sixty-three days old at the beginning of the experiment and, from the work of Pearl ('21) on the longevity of flies, we could infer that a few of the individuals might have been from the very first emergence.

RECOVERY AFTER EXPOSURE TO CARBON DIOXIDE, HYDROGEN, AND NITROGEN CAUSING AN ASPHYXIATION CONDITION SIMILAR TO IMMERSION IN WATER

The effect of various gases in causing asphyxiation was also tried as a comparison with immersion in water, using carbon dioxide, hydrogen, and nitrogen for this study. The CO_2 and H_2 were produced in a Kipp generator, washed with H_2O, and then the containers with the flies were flooded with the gas. The N_2 was taken from the compressed storage cylinder supplied by the Matheson Company, and was also washed through distilled water. In order to have comparable results, it was found necessary after a fifteen-minute exposure to the gases to immerse the flies in water for one minute, thereby thoroughly wetting their wings before spreading them out on the moist paper toweling; otherwise, many

of the flies on recovery would 'death feign' and begin flying without preliminary warning, such as leg movement which would usually precede attempts to fly after immersion in water. Also it seemed desirable to have these flies, as far as their external environment was concerned, in the same conditions as the first group of flies. Experiments were made on three groups of flies by exposing them to the gases. The three-day, the five- and six-day, and one group of old flies were observed, and in all cases the curves of recovery correspond very closely with curves of recovery from immersion in water; and also, the egg-laying records show a similarity, although there is more variability in the rate of egg laying. Only one figure, figure 5, is included from this series of experiments illustrating the effect of H_2 gas. This curve again illustrates the first group or the three-day old flies. The mean recovery time of the male was 14.6 and of the female 15.0 minutes, with probable errors of $\pm$ 0.2379 and $\pm$ 0.1503. Here again the egg-laying record per female per day shows the peak to have been at the twenty-minute period for the first eight days, with a gradually falling toward the earlier five-minute period. A typical egg-laying curve of the first type resulted for the first eight days; later, when yeast was added to the sterile culture medium, as was done on the last two days, an enormous increase in egg laying occurred. Within one day the normal symmetry which was established as an effect of immersion in water or exposure to gas had been changed, some individuals in the early recovery period having their gonads stimulated to a greater extent than the later-period individuals. The erratic shift of the egg-laying curve is very characteristic of flies fed daily on fresh yeast, as is pointed out later on in detail.

The egg-laying curve of individuals which have not been subjected to immersion in water or gas (fig. 6) is made up of two experiments: A includes flies from a culture six days old; B, from a culture three days old. In each case the flies were etherized for one-half minute and by random sampling five females were selected and placed in each of the four

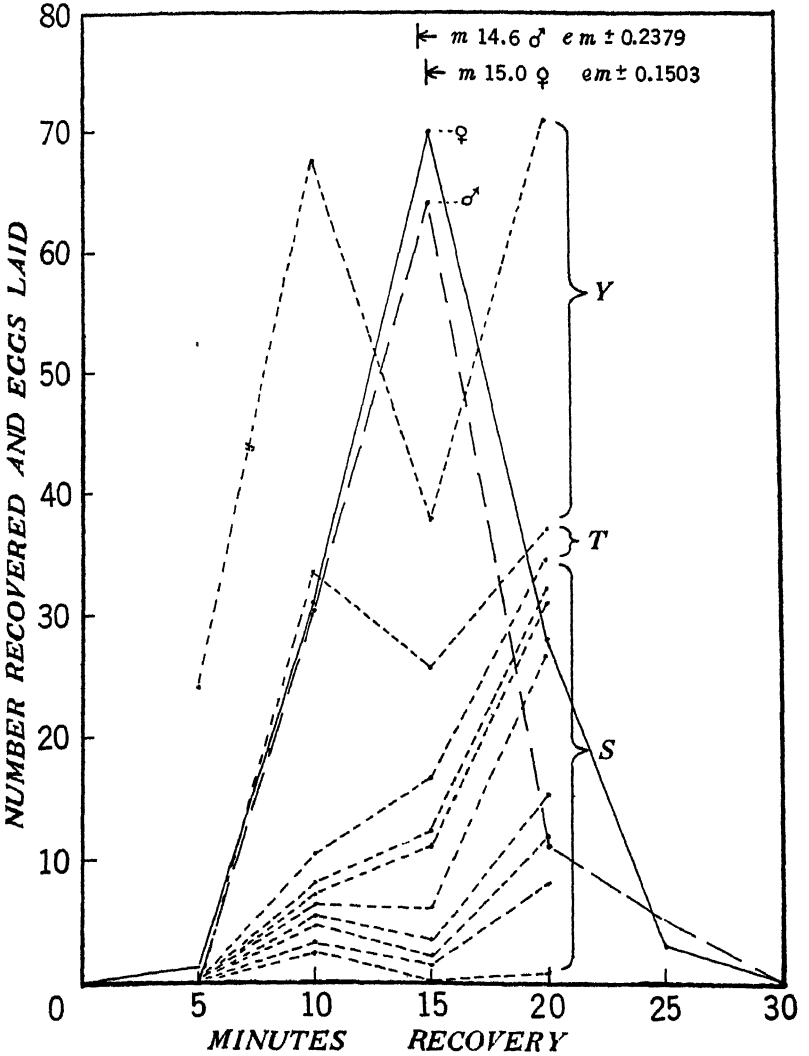

Fig. 5 Curve of egg laying of this group for a period of eight days is of the first type, in which females recovering late lay more eggs than those recovering early (dotted line); recovery from asphyxiation by H_2 gas is shown by solid line for females and broken line for males. *Y*, yeast-fed adults; *T*, temperature change from 25° to 30°C.; *S*, sterile medium. Age of this culture was three days. *m*, mean; *em*, probable error.

egg-laying trays. The curve of egg laying for the first few days approaches a straight line with slight deviations. The addition of yeast causes some individuals to go far ahead of others in the development of gonads, as indicated by egg laying during the last three to four days.

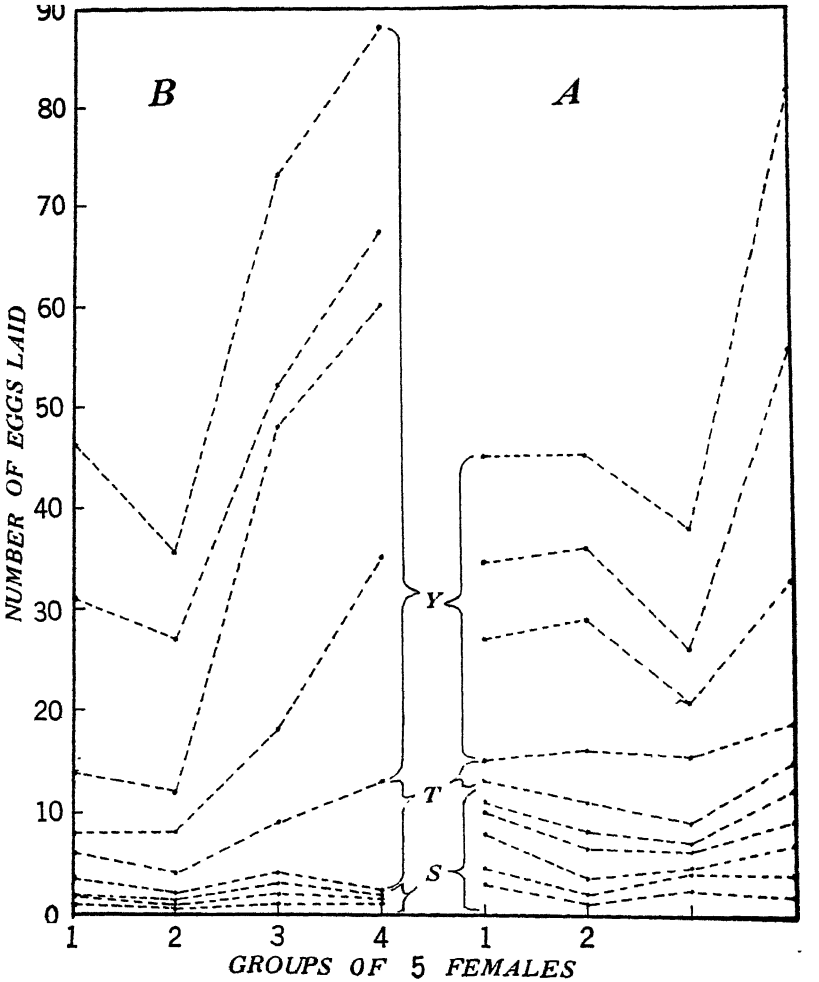

Fig. 6 This graph represents Drosophila flies etherized, and by random sampling five females were selected for control experiments in egg laying. *A*, six days old; *B*, three days old; *Y*, yeast fed to adults; *T*, temperature increase from 25° to 30°C.; *S*, sterile medium.

EXPERIMENTS WITH 'VESTIGIAL' FLIES

Since several workers, including Bodine and Orr ('25), found that there is a difference between 'wild' and 'vestigial' flies, several experiments were performed on vestigial flies in the second group—namely, the five- and six-day old—in order to throw light on the physiological differences between these two types of flies. The results of the experiments on recovery from immersion in water are illustrated in figure 7 and are similar to those of the normal flies, with

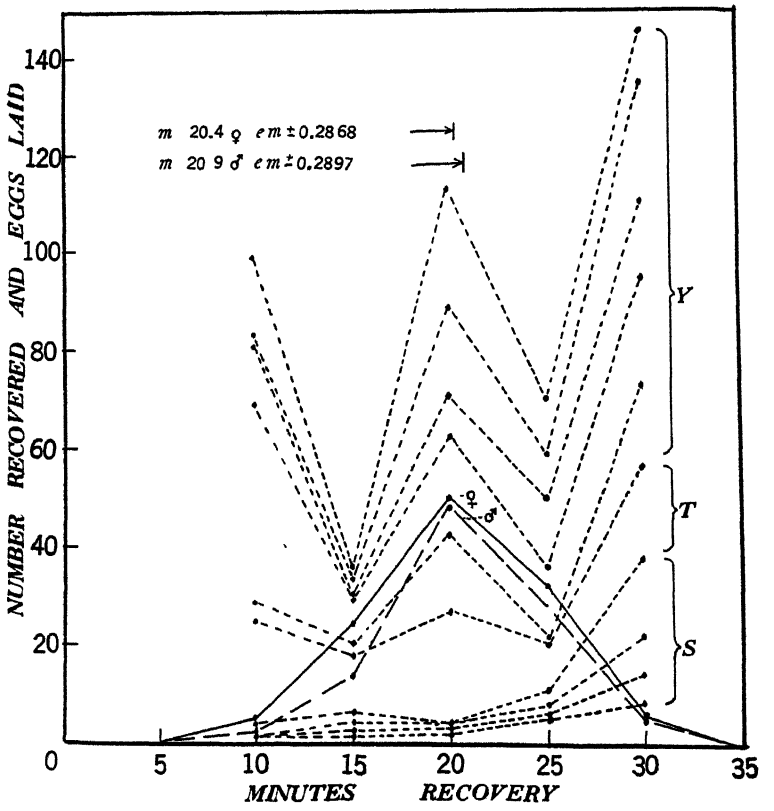

Fig. 7 'Vestigial' graph of recovery from immersion in water (solid line, females; broken lines, males; dotted lines, egg-laying per individual per day). Culture six days old. Egg-laying curve of the first type. *Y*, yeast fed to adults; *T*, temperature increase from 25° to 30°C.; *S*, sterile medium; *m*, mean; *em*, probable error.

a mean of the males as 20.9 minutes and females as 20.4 minutes and the probable errors $\pm$ 0.2897 and $\pm$ 0.2868.

The egg laying of individuals in these groups of vestigial-fly experiments was less per individuals per day than in the normal flies as frequently observed. Also the type of egg-laying curve was not as expected, in that the highest egg laying occurred from flies at the end of the recovery period, as shown in figure 7. This figure also shows the enormous increase in egg laying when the adult flies were fed on yeast and also the tendency of the curve to deviate from the previously secured curve with the addition of a strong gonadal stimulation.

EGG LAYING, SEX RATIO, AND PER CENT EMERGENCE

It was thought advisable to secure the percentage hatching of these five-minute periods of daily egg laying, and numerous attempts to rear the flies through to maturity universally resulted disastrously in all culture dishes when the number of eggs was few. Mold and bacteria were found to grow faster than the larvae and interfere with their development. However, if there are numerous larvae, they will keep down the growth of mold and bacteria which is bound to occur, since the adults were not sterilized.

Hyde ('24) gives figures relative to the fertility of wild flies and closely inbred flies. The fertility of the entire group of wild flies studied by him averaged 89 per cent, while the fertility of the closely inbred flies was 57 per cent. Lutz ('14) states the egg laying per day as being 6.7 for each fly and Adolph ('20) gives the figures as 3.3 eggs per day and the maximum number as thirty-three eggs per day. Guyénot ('13 g), who obtained twenty to twenty-five eggs per day, also points out that poor nutrition decreases egg laying. Adolph ('20) states that egg laying is stimulated by a combination of moisture, texture, and odor, and that it is an 'all or none' response. Barrows ('07) concludes that food is more tropic than odor. Guyénot ('07) first recognized yeast or bacteria as necessary to larval development. Guyénot

('07) and Baumberger ('19) speak of the rôle of yeast as a loose symbiosis. Loeb ('15) states that yeast induces egg laying. In order partially to check up these results and also to gain an idea of the progressive decrease in fertility, since Hyde claims that the fertilization of the entire egg string does not occur as a result of one mating, each day's egg laying of the entire group was placed in a common sterile culture bottle and the emergence noted. During the first eight days the eggs laid were all derived from the same series of experiments; the decrease in number of females was principally due to death and loss of a few individuals. On the ninth, eleventh, twelfth, and fourteenth days, additional experiments were started which increased the number of females.

Table 1 can be arranged under four headings; first, eggs laid by females on sterile medium and yeast not fed to larvae; secondly, eggs laid on sterile medium and later yeast fed daily to larvae; thirdly, the same as the second, excepting the temperature during the one day of egg laying was 30°C. instead of 25°C.; and, fourthly, yeast was fed daily to adults and larvae. The eggs per day per female and the emergence are illustrated in figure 8 and show the stimulating effect of yeast on egg laying, the depression of emergence of the adult as a result of immersion in water, and suggest that the entire egg string is probably not fertilized at one mating.

EGG LAYING AS AN INDEX OF METABOLIC RATE AS SHOWN BY DIFFERENCE IN O_2 CONSUMPTION

Higher O_2 consumption in flies which were more actively laying eggs as contrasted with similar groups which were laying fewer eggs indicates that the active egg-laying flies have a higher metabolic rate.

Six hundred and ninety-five flies of known age varying from two to seventeen days old were isolated in groups of from ten to twenty flies and were fed yeast; also, 655 flies were similarly treated except that they were not fed yeast. The O_2 consumption of comparable yeast versus no-yeast

weighed groups was secured by placing ten to twenty yeast-fed flies of known age in a Krogh manometer modified by Bodine and readings taken for two hours; then the flies were removed and the weighed groups of no-yeast-fed flies were placed in the same manometer and O_2 consumption recorded. The number of eggs laid by each group of flies was also known. Table 2 and figure 9 give the comparison of yeast versus no-yeast-fed flies. First, in comparing the number

TABLE 1

Eggs laid on sterile medium and larvae not given yeast

DAY	EGGS LAID	NUMBER FEMALES	EGGS PER FEMALE	EMERGENCE		TOTAL	PER CENT EMERGENCE (FERTILITY)
				Male	Female		
1	1025	155	6.6	57	63	120	11.7
2	694	132	5.2	103	97	200	28.8
3	479	118	4.0	141	142	283	59.1
4	600	118	5.1	149	154	303	50.5
5	343	117	2.9	78	73	151	44.0
6	324	116	2.8	56	76	132	40.7
7	242	111	2.2	49	47	96	39.6
8	161	93	1.7	41	39	80	49.7
Total	3868	960	4.0	674	691	1365	35.3
Eggs laid on sterile medium plus new stock of experimental flies on ninth, eleventh, twelfth, and fourteenth days and yeast given to larvae							
9	298	206	1.4	75	81	156	52.4
10	297	180	1.6	80	86	166	55.9
11	278	140	2.7	128	147	275	72.8
12	456	204	2.2	103	111	214	46.9
13	285	213	1.3	125	118	243	85.2
14	488	192	2.5	85	87	172	35.3
Total	2202	1135	1.9	596	630	1226	55.7
Temperature increased from 25°C. to 30°C. and additional experimental flies added							
15	944	187	5.0	194	214	508	53.8
Fleischmann's yeast fed to adults and larvae							
16	2658	166	16.0	107	130	237	8.9
17	1957	148	13.2	246	270	516	26.4
18	1401	87	16.1	104	114	218	15.5
19	816	48	17.0	40	37	77	9.5
20	670	35	19.1	25	16	41	6.1
Total	7502	484	15.5	522	567	1089	14.5

of etherizations and manometer readings of each group, no group of flies was etherized more than six times. The second comparison shows the per cent increase of egg laying due to yeast. In the third place, the same table and graph show the

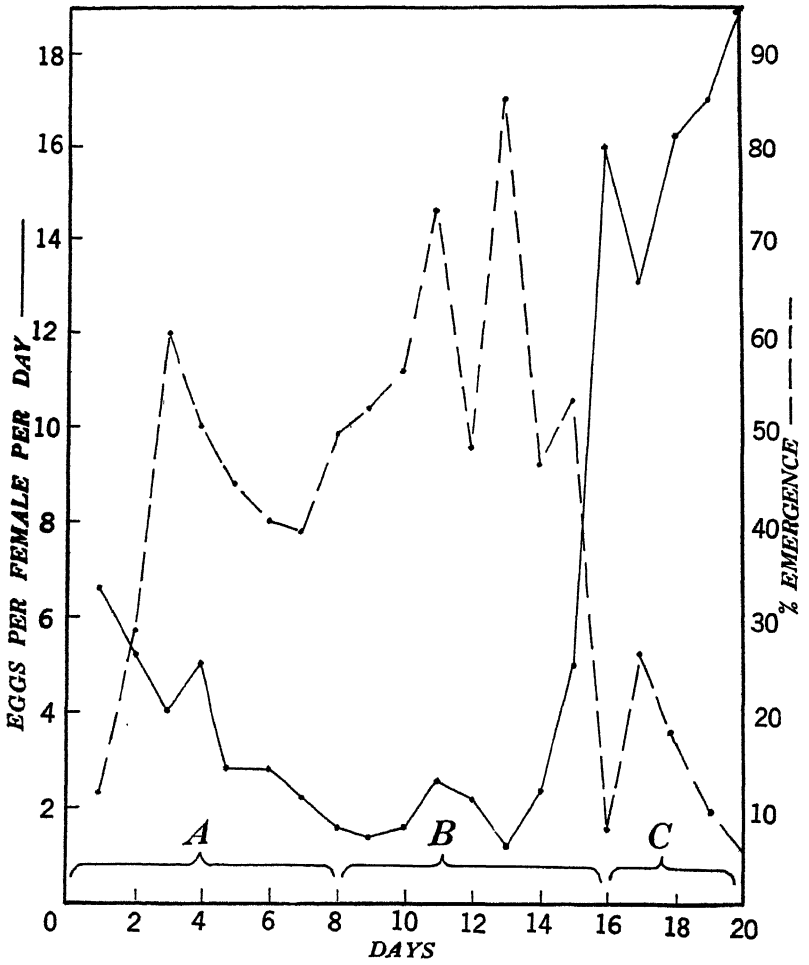

Fig. 8 The solid line represents the eggs per female per day over a period of twenty days and the broken line gives the percentage of emergence of adults. *A*, no yeast fed to larvae or adults; *B*, yeast fed to larvae; *C*, yeast fed to larvae and adults. First day has a low per cent of emergence, due to immersion in water. The low per cent of emergence of the last few days was due to the eggs not being fertile.

per cent increase in weight. Fourthly, comparing one group of ten yeast-fed flies with a similar number of non-yeast-fed flies, the per cent increase of O_2 consumption is shown; the table and corresponding graph indicate the same also in terms of gram weight.

Table 3 is an analysis of the data in column 1 of table 2 and also gives the age of each group of flies at the time the

TABLE 2

Per cent increase of eggs, weight, and O_2 consumption due to feeding yeast. Experiments combined regardless of age of flies. Column 1 represents the first etherization and manometer readings, column 2 represents the same groups examined the second day, and the remaining columns, continuation for subsequent days

	FOOD	1	2	3	4	5	6	AVERAGE
Eggs per 10	Yeast	176	209	162	140	155	133	975
flies	No yeast	111	90	29	97	132	111	570
Increase, per cent		58.5	132.2	45.8	44.3	17.44	19.8	71
Weights per	Yeast	.0103	.0098	.0129	.0086	.0105	.0089	.0610
10 flies	No yeast	.0100	.0089	.0097	.0065	.0085	.0078	.0514
Increase, per cent		3	10.1	32.9	32.3	23.5	14.1	18.6
Manometer	Yeast	26.9	19.9	23.6	16.6	12.9	7.9	107.8
readings	No yeast	22.7	15.5	16.5	10.8	8.6	7.0	81.1
Increase, per cent		18.5	28.3	43	53.7	50	12.8	32.9
Gram weight O_2	Yeast	2412.8	1742.8	1762.3	1853.8	1224.8	824.8	9821.3
consumption	No yeast	2200.9	1548.9	1727.0	1242.8	848.2	794.4	8362.2
Increase, per cent		9.6	12.5	2.0	49.1	44.4	3.7	17.4

O_2 consumption was determined, and the treatment of the flies was as follows: flies one day old were separated into two groups, one fed yeast and the other not; these were considered as experimental stock bottles, and if any yeast infection occurred, it would be by the flies carrying it in or on their bodies. The day before the O_2 was to be determined, these flies were transferred to fresh bottles—the original yeast-fed flies to fresh bottles with yeast and the no-yeast-fed flies to bottles without yeast.

DISCUSSION

Flies which are three days old recover from immersion in water and the effect of gases, CO_2, H_2, and N_2, in less time than flies which are older. That is shown by an advance of the mean of 4.62 minutes with a probable error of $\pm$ 0.498 for males and mean of 4.12 for females $\pm$ 0.413, which is a significant difference. The flies raised on old culture medium

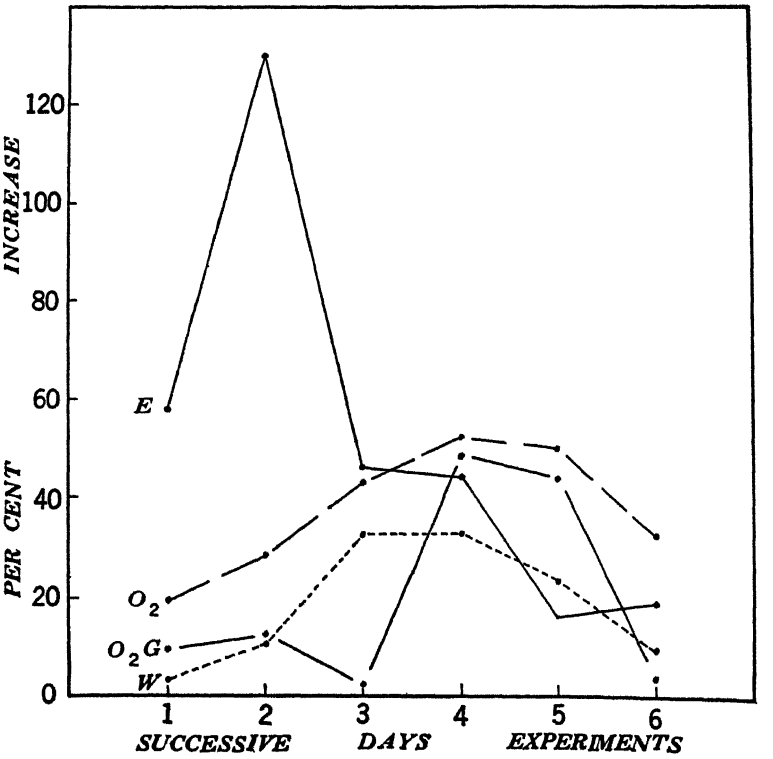

Fig. 9 Egg laying as an index of metabolic rate as shown by increase in O_2 consumption. All comparisons show the per cent increase of yeast-fed flies over the non-yeast-fed flies on successive days of experiments regardless of age. *E*, per cent increase of eggs laid by flies fed yeast compared to similar groups to which yeast was not fed; *W*, weight increase of yeast-fed versus non-yeast-fed flies; O_2, the per cent increase of O_2 consumption of flies fed yeast as contrasted with flies to which yeast was not fed. This is given on a ten-fly basis. O_2G, the per cent increase of O_2 consumption calculated in terms of per gram weight of fly.

have a difference from the five- to six-day-old groups of 3.65 minutes, with a probable error of $\pm$ 0.687 for males and of 5.10 minutes for females with a probable error of $\pm$ 0.945, but these differences are not significant.

TABLE 3

Per cent increase or decrease of eggs, weight, and O_2 consumption due to feeding yeast as is summarized in table 2, column 1

AGE, DAYS	EGGS PER 10 FLIES		PER CENT	AGE	WEIGHT OF 10 FLIES		PER CENT
	No yeast	Yeast			No yeast	Yeast	
2	0	0		2	.0089	.0042	—52.8
2	0	0		2	.0050	.0057	14.0
6	32	52	63.1	6	.0118	.0118	0.0
9	142	291	34.5	9	.0081	.0103	36.8
10	91	257	18.3	10	.0111	.0107	— 3.6
11	152	160	5.2	11	.0111	.0112	11.1
12	241	254	5.8	12	.0132	.0107	—11.3
14	170	180	5.8	14	.0092	.0121	31.5
15	151	230	45.7	15	.0085	.0125	47.0
15	93	223	139.8	15	.0117	.0119	58.5
16	68	221	22.5	16	.0094	.0095	10.5
17	130	235	80.7	17	.0114	.0121	16.2
18	179	196	9.5	18	.0109	.0114	45.9
Average	176	111	58.5		.0100	.0103	3

O_2 consumption as shown by:

AGE, DAYS	O_2 CONSUMPTION IN CU.MM. PER 10 FLIES PER ONE HOUR		PER CENT	PER CENT PER GRAM WEIGHT
	No yeast	Yeast		
2	20.73	22.17	6.8	128.2
2	20.97	20.97	0.0	— 12.4
6	50.61	74.71	47.6	48.5
9	45.80	47.00	2.6	— 15.1
10	52.78	63.86	21.4	31.7
11	64.59	55.19	—14.5	— 15.5
12	68.93	93.03	34.9	75.4
14	60.73	56.15	— 7.5	— 32.9
15	75.67	77.84	— 2.8	— 46.2
15	71.34	86.76	21.6	19.4
16	61.70	84.35	36.7	35.1
17	66.76	84.35	26.3	45.2
18	53.02	77.60	46.3	39.8
Average	54.71	64.83	18.5	9.6

Probable error of per cent increase of eggs, no-yeast versus yeast-fed flies = 0.002. Probable error of per cent O_2 consumption = 0.011.

In all experiments conducted only in one instance was the mean of the males more than two minutes different from the females. In three instances the mean of the males was earlier than the mean of the females, by an average of 0.7 minute; and in fourteen cases the mean of the male was later than that of the female by an average of 0.97 minute, with probable error of $\pm$ 0.542. While this is not a significant retardation, it is nevertheless of some importance in indicating that the females may have a slightly higher metabolic rate than the males.

Egg laying in flies recovering during five-minute intervals falls under three types; first, three-day females from new cultures, in which case females toward the end of experimental periods lay more eggs than those from the early recovery period; secondly, flies which are five or six days old fall in a rapidly decreasing curve, the first group of flies which emerge in the ten- or the fifteen-minute period laying more eggs than those that recover at late periods; thirdly, old cultures without larvae have a very low egg-laying rate throughout the entire time and those that recover near the end of the experiment at thirty, thirty-five-, and forty-minute intervals are practically spent individuals. According to Riddle ('27), when pigeons are at the height of their egg-laying activity, their metabolic rate is the highest.

The effect of yeast in food causes an increase in egg laying, as shown by table 2 and figure 9 to be 71.0 per cent. In the case of the flies the yeast increased their weight 18.6 per cent and the O_2 consumption was increased 32.9 per cent. The variance, when calculated in terms of gram weight, was 17.4 per cent. This can be accounted for by the variability of yeast consumption which causes the weight relationship to be irregular; therefore, one would not be justified in comparing metabolic rates on the basis of weight only as a starting-point unless the flies were fed a meager constant diet, in which case erratic stimulation would not occur. This is shown by the comparison of the age groups in table 3, where, in two cases, the yeast-fed flies had a lower O_2 consumption

than the no-yeast-fed flies as contrasted with flies on the ten-fly basis, and can be accounted for by a carrying of yeast by flies not fed yeast into a medium which did not have yeast. Upon subsequent transfers to a fresh medium, the contamination is gradually eliminated and therefore the O_2 per cent difference between flies fed yeast versus flies not fed yeast becomes greater. Therefore, a definite correlation can be demonstrated in the case of insects, showing that when insects have a higher egg-laying rate they have a higher metabolic rate as judged by O_2 consumption, as compared with other insects of the same age in which the egg-laying rate is less per fly.

Since five- and six-day-old flies recovering from immersion in water in five-minute intervals of time are separated into a graded series of egg-laying capacities, one would infer that those recovering late are of a lower metabolic rate, since they lay fewer eggs. The three-day-old group would not fit in with this conclusion, because those which recover first are younger from the standpoint of their gonads and therefore those which recover later are more mature and have a higher capacity for egg laying. The earlier mean of the first group would also indicate that a more rapid metabolic rate occurs in individuals which are younger as contrasted with older individuals. If the mean of periods of recovery can be taken for an index of metabolic rate, then we would expect 'vestigials' to recover in less time than the normal flies. The fact, however, that the egg-laying rate of the six-day-old group of flies has a tendency to be more like the curve of egg laying of normal three-day group would point to a younger state in 'vestigials' than in the 'wild' flies. The six-day group of 'vestigials,' as far as egg laying is concerned, would be comparable to three-day normal flies.

There is no apparent difference in flies which recover from immersion in water as compared with those treated with CO_2, H_2, and N_2 gases. The means fall in the same general range as the immersion-in-water mean and egg laying is about the same; there is, however, a little more tendency to irregularity in the egg-laying rate.

The first eggs laid by females after immersion in water are far less fertile than those laid a day later, as shown by using emergence of adults as an index of fertility, and illustrated by the fact that the first day adults emerged from 11.7 per cent of 1025 eggs; the second day, 28.8 per cent of 694 eggs, and the third day, 59.1 per cent of 479 eggs. The fertility reaches a maximum on the third day and then falls off toward the end of the eighth day. This would substantiate Hyde's claim relative to fertility.

Yeast has been known to be necessary for larval development, and although Guyénot has raised forty successive generations of flies entirely on sterile medium, the percentage yield of adult flies was very low.

No statement seems to have been made relative to quantitative increase of egg laying due to yeast in the adult except that Loeb states that it stimulates egg laying. Table 1 shows than in eight days 120 adults laid 3868 eggs, or 4.0 eggs per day per fly, on sterile medium; in a later six-day period 189 females laid 2202 eggs, or 1.9 eggs per day for each fly. In a later period of five days 97 females laid 7502 eggs when 0.05 cc. of Fleischmann's yeast solution (one-eighth cake to 10 cc. water) was added daily to each sterile egg-breeding chamber. This would indicate an increase in egg laying of 375 per cent, comparing the egg laying of the flies fed on yeast with the flies which were not fed yeast for eight days, with the 816 per cent increase of egg laying, comparing the egg-laying of the sixteenth to twentieth day with the egg laying of the ninth to fourteenth day. Guyénot ('13 e) secured an increase of about 400 per cent of egg laying with Drosophila when fed yeast versus sterile potato. Yeast therefore is a very important factor in stimulating egg laying in Drosophila. The emergence of adults as an index of fertility is also shown in the table as being 6.1 per cent on the last day, which is a marked decrease in fertility of eggs and is due largely to the fact that the entire egg strings were not fertile.

The per cent increase of larvae living when yeast was added to diet was 20.4 per cent. Yeast therefore seems to

be a more stimulating factor in egg laying of the adults than in growth of larvae.

Adolph ('20) gives the greatest egg laying per day per fly as thirty-three, while in the experiments shown in table 1 the greatest egg laying per day per fly was fifty-two when yeast was fed daily, and this was in an egg-laying chamber containing three females. Therefore, the egg laying of a single fly may have been considerably higher.

Immediately upon the addition of the yeast to the culture medium, all of the flies would settle down around the drop and feed for several minutes. Guyénot ('13 e) speaks of insects belonging to three groups in considering the development of gonads; first, those in which the state of the gonads depends entirely on the nutrition of the larvae, as, for example, in certain Lepidoptera; secondly, insects in which the larva, pupa, and adult influence state of gonads, as in Drosophila; and thirdly, insects in which the nutrition of the adult affects the gonads, as in Calliphora.

It appears, therefore, that the feeding of yeast in Drosophila affects the gonads and any study of egg laying requires that the food factor be carefully considered in the larvae and adult in order to insure uniform results.

Drosophila is capable of retarding the laying of eggs if food conditions are adverse, as when fed sterile non-nutrient medium such as potato, carrot, etc., to the extent that occasionally young larvae are born instead of eggs being laid and the insect then becomes temporarily viviparous; this is in accordance with the findings of Guyénot ('13 g).

Retardations in individual egg laying account in part for variation in fecundity of Drosophila.

RECOVERY FROM IMMERSION IN WATER OF THE JAPANESE BEETLE

A comparison of the effect of immersion in water on recovery of a dipterous insect, Drosophila, was made with a coleopterous insect, the Japanese beetle.

The time factor in the recovery of the Japanese beetle (Popillia japonica Newm.) from immersion in water can be

used as an index of the physiological state of the insect and also the condition of the gonads in both males and females.

On the 12th of August, sixty-eight females and seventy-eight males were submerged in water for fifteen minutes at 25°C. These beetles were previously divided into six lots

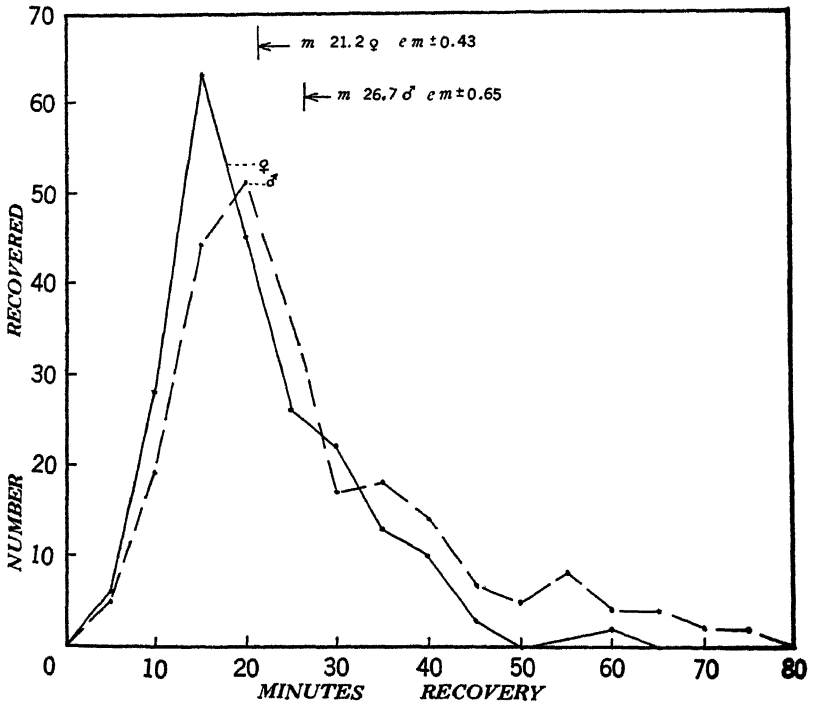

Fig. 10 Recovery from immersion in water of the Japanese beetle. The solid line represents 224 females and the broken line, 231 males. This shows the combination of August 12 and September 8, 1927, experiments. *m*, mean; *em*, probable error.

and given three kinds of leaf diet for twenty-four hours; namely, apple, geranium, and blackberry. The beetles were placed on filter-paper and as recovery occurred they were transferred to jars at the end of each five-minute period.

On the 8th of September, a similar series of experiments was run on 156 females and 153 males, divided into eight lots and fed on apple, geranium, smartweed, and sassafras. Dur-

ing the latter part of the summer smartweed is one of the principal plants on which the beetles were normally feeding.

The plotted curves of recovery time show that they fall in a typical U curve, with the maximum recovery of either sex a little to the left of the mean.

An examination of the frequency curves (fig. 10) of recovery, which are a combination of two sets of experiments

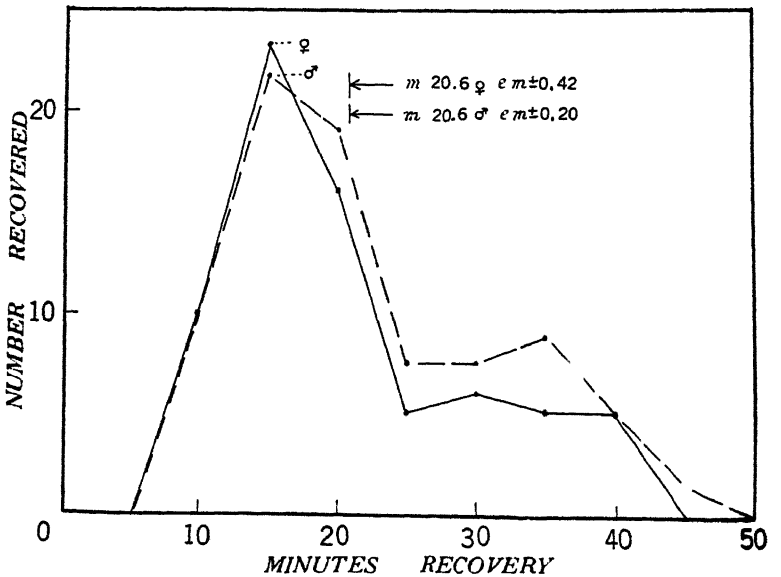

Fig. 11 Frequency curve of recovery from immersion in water of the Japanese beetle on August 12, 1927. The sixty-eight females are represented by a solid line and the seventy-eight males by a broken line. *m*, mean; *em*, probable error. Since the mean of the males and the females is the same, both sexes probably have the same metabolic rate.

of 224 females and 231 males, August 8th and August 12th, indicates that there is an apparent retardation of the mean of recovery of 5.5 minutes of the males as compared with the females, with a probable error of $\pm$ 0.65. If, however, these records are plotted according to the month when experiments were made, a contrasting difference is to be noted.

Figure 11 gives the record of the August 12th recovery, and in this case the mean for the females and males is 20.6

minutes, indicating that at this time of the year the metabolic rate and reserve oxygen debt supply are about equal in the sexes. On September 8th the frequency curve of recovery (fig. 12) shows that the males have a lower metabolic rate in that the mean is 21.5 minutes for the females and 29.1 for the males, or a retardation of 8.1 minutes, and with the probable error of females being $\pm$ 0.57 and males, $\pm$ 0.87. Due weight should be considered for this late recovery.

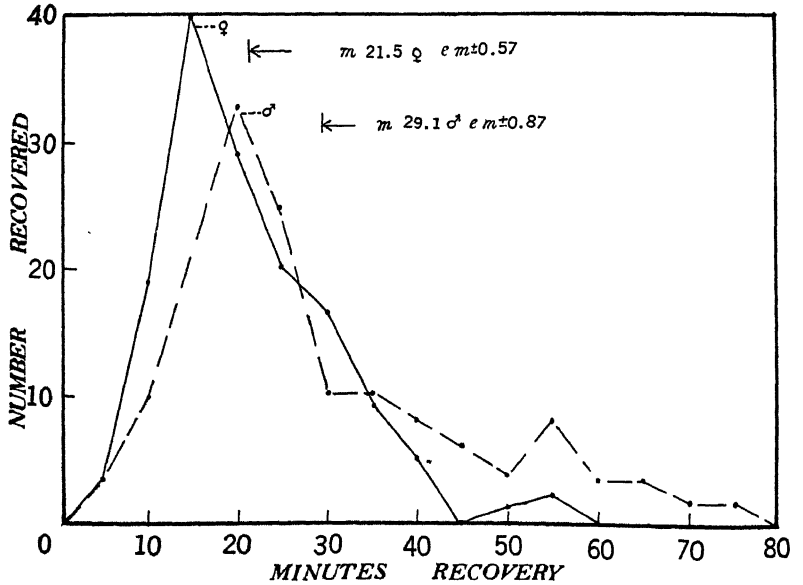

Fig. 12 This graph illustrates the recovery from immersion in water of the Japanese beetle on September 8, 1927. Solid line represents 156 females and the broken line, 153 males. *m*, mean; *em*, probable error. Since the mean of the males is much later than that of the females and since the males were found to be spent individuals, the metabolic rate of the males would probably be lower than that of the females at this period of the year.

Comparing this August recovery with the September recovery, the difference of 0.9-minute recovery of females is insignificant. The mean of the frequency curves of the male, being 20.6 minutes in August and 29.1 minutes in September, indicates a difference of 8.5 minutes, which would show a slower metabolic rate for the males in August as contrasted with September.

Beetles from these different experiments were dissected and the conditions of the gonads noted. In the August group of beetles the ovaries of the beetles recovering after from twenty to twenty-five minutes did not contain large eggs and the spermaries appeared spent. The beetles experimented on in September did not have large eggs in those which recovered after from twenty-five to thirty minutes, and the spermaries of the males which recovered from twenty-five to thirty minutes appeared spent.

The rate of recovery from immersion in water in Japanese beetle, using insects where the egg-laying period is prolonged, offers a method of selecting the spent individuals from those which will lay eggs within a short time, and this method could be used in determining the value of baits and traps in selecting individuals for experimental work on insects in the same metabolic state.

CONCLUSIONS

1. Recovery periods from immersion in water can be used to indicate the metabolic rate of Drosophila melanogaster Meigen and Popillia japonica Newm. In Drosophila the mean of recovery time from immersion in water is retarded in the males by 0.97 ± 0.542 minute from the females, indicating a slightly lower metabolic rate for the males. The Japanese beetle in August, having the same mean of recovery for both sexes from immersion in water, would show them probably to be of equal metabolic rate in that month and the 8.1-minute shift of the mean for the males in September would indicate that males are in a more depressed physiological condition than the females.

2. Recovery periods from immersion in water also can be used to divide flies into groups possessing approximately equal egg-laying capacities. In this manner spent individuals can also be isolated from others still capable of egg laying.

3. Yeast appears to be more of a stimulant to egg laying in adult flies than it is to larval growth.

4. Flies exposed to carbon dioxide, hydrogen, and nitrogen are asphyxiated, similar to immersion in water.

5. 'Vestigial' flies recover from immersion in water in a manner similar to 'wild' flies.

6. Yeast-fed flies have a higher metabolic rate as judged by greater O_2 consumption than non-yeast-fed flies.

ACKNOWLEDGMENT

The stock cultures for these experiments were obtained through the kindness of Dr. R. L. King. The 'wild' flies were collected by him at Nescopec, Pennsylvania, four years ago, and the 'vestigials' which were supplied by him originally came from Columbia University.

To the following students, H. C. Wimmer (Pennsylvania) and R. A. Lumley (Dickinson), the author is indebted for assistance in collecting data.

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LIFE CYCLE, SEX DIFFERENTIATION, AND TESTIS DEVELOPMENT IN MELANOPLUS DIFFERENTIALIS (ACRIDIDAE, ORTHOPTERA)

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TWO TEXT FIGURES AND FIVE PLATES (FIFTY FIGURES)

AUTHOR'S ABSTRACT

The general morphological and histological evidence accumulated by this study suggests the following facts.

1. Under out-of-door conditions, in the vicinity of Philadelphia, Pennsylvania, embryonic development begins at deposition and continues to the middle or late spring, when hatching occurs. The postembryonic development is completed during the summer. Copulation and oviposition occur in the late summer and early fall.

2. The embryonic development may be divided as follows:

a. The prerevolution period, in which the rudiments of organs and systems are formed.

b. The early-revolution period, during which the direction of the embryo in the egg is reversed.

c. The late-revolution period, or time of yolk circumcrescence and completion of the dorsal wall of the embryo.

d. The postrevolution period, comprising development from yolk engulfment to hatching.

3. The sexes are differentiated during the early- and late-revolution periods.

4. In the differentiation of the genital rudiments, *a*) the germ cells are segregated into groups; *b*) an indifferent mesodermal element grows in among the germ cells of such a group; *c*) the processes of this cell (the apical cell) form intimate connections with the processes of connective-tissue elements surrounding the germ-cell group; and, *d*) the covering membrane of the genital rudiment grows in between the various germ-connective-tissue cell groups, completing the rudiment of the follicle.

5. When the adult condition is reached the testis is functionally differentiated.

CONTENTS

Introduction 468
A. The problem 468
B. Material and methods 469
C. Acknowledgments 471
Observations 471
A. Life history 471
1. Oviposition 471
2. Development within the egg 471
a. Prerevolution period 472
b. Early revolution period 474
c. Late revolution period 477
d. Postrevolution period 478
3. Hatching and the pronymph or vermiform larva 478
4. The instars 479
5. The adult 479

B. Sex differentiation 480
1. External genitalia 480
2. The genital rudiments 481
C. The development of the gonad 481
1. The early genital rudiments 481
a. The terminal filament 482
b. The dorsal cell mass 482
c. The central cell mass 482
d. The ventral cell strand 483
e. The outer limiting membrane 483
f. Isolated germ-cell groups 483
2. The genital rudiment during the latter part of the revolution period 483
3. The genital rudiment at the beginning of the postrevolution period 485
4. The genital rudiments at the middle of the postrevolution period 486
5. The genital rudiments at the time of hatching 487
6. Apical cell formation 489
7. The formation of the follicle rudiment 490
8. The developing gonads at the time of the second instar 490
9. The differentiation of the ventral cell strand and germinal center 493
10. The formation of the spermatocysts 497
11. Spermatogenesis in relation to the instars 499
12. The growth of the follicles 500
13. The differentiation of the follicle ducts 500
14. The adult testes 500
Summary and discussion 501
A. Summary 501
B. Discussion 503
Literature cited 509

INTRODUCTION

A. The problem

This study has had the germ-cell history and the origin of the apical cell for its center of interest.

The germ-cell history is important for an understanding of the relation between spermatogenesis and oogenesis (McNabb, '28). There are involved here the origin of the germ cells, the differentiation of sex, and the development of the male and female gonads.

The origin and function of the apical cell, in relation to the germ cells, has aroused considerable interest. It has been

described in the germ glands of various insects. In the Lepidoptera: Verson ('89, '94), Cholodkovsky ('94), Grünberg ('03), Munson ('06), and others. In the Diptera: Cholodkovsky ('92 and '05). In the Coleoptera: Holmgren ('01). In the Orthoptera: Davis ('08), Gerard ('09).

Regarding the origin of this cell, some—e.g., Grünberg—have come to the conclusion that it is a modified germ cell, while Toyama ('94) believed it to be a 'follicular cell.' Its origin, therefore, is open to question, as is also its function.

The investigation of the germ-cell history in the grasshopper has entailed a general knowledge of its embryology, regarding which references in the literature are very meager and unsatisfactory. Observations upon the developmental cycle were necessary as a part of the plan of this investigation.

B. Material and methods

The form used for this study is the grasshopper, Melanoplus differentialis. It was selected because the eggs are easily obtained in sufficient numbers and the nymphs and adults are readily maintained under laboratory conditions. The embryos were taken from eggs which were allowed to develop under out-of-door conditions in small boxes of moist sand or from eggs that were incubated at a temperature of 25°C.[1]

The individuals studied during the postembryonic development were isolated immediately after the intermediate or pronymphal molt, and kept in small individual cages upon a diet consisting mainly of lettuce. The molts of each individual were noted. The number of antennal joints, the external genitalia, and wing development were used as a set of criteria for the instars.[2]

The solutions employed for the fixation of embryos were Carnoy-Lebrun and B_3. Embryos fixed in Carnoy-Lebrun

[1] Regarding the care of hoppers in the laboratory, see Carothers ('23).

[2] The writer is preparing for publication his observations on the nymphal changes in Melanoplus differentialis.

were controlled with a microscope. When the embryo became opaque, the egg was removed, washed in several changes of 70 per cent alcohol, and finally preserved in 70 per cent alcohol. When B_3 was used, the egg membranes at the ends of the egg were removed and the egg immersed in the fixative for twenty-four hours or longer. All material from the time of hatching to the adult was fixed in B_3, B_3 plus three drops of 50 per cent chromic acid to each 5 cc. of fixative, or bichloride-formol.

The embryos and younger nymphs were cleared in oil of cedar wood, washed in benzol, and infiltrated in two changes of paraffin. Aniline oil was used as the clearing agent for all other material. The staining solutions utilized were Heidenhain's iron-hematoxylin, Heidenhain's iron-hematoxylin followed by v. Gieson, Mallory's triple stain, and orcein.

Aside from the numerous individuals sectioned for observations on the earlier embryonic development and the individuals observed in toto during various stages of the life cycle, the following individuals were sectioned and utilized for observation on the developing gonad from the time of sex differentiation to the early adult condition.

11 (males and females)[1]	during the early revolution period
15 (males and females)	during the late revolution period
43 (males and females)	during the postrevolution period
12 (males)	at the time of hatching
16 (males)	during the first instar
10 (males)	during the second instar
9 (males)	during the third instar
11 (males)	during the fourth instar
8 (males)	during the fifth instar
15 (males)	during the sixth instar
9 (males)	adults

[1] As denoted by the external genitalia.

The majority of the individuals sectioned during the revolution, postrevolution, and early first-instar periods were sectioned entire, whereas in the case of the individuals from the middle of the first instar to the early adult condition, only the region containing the developing gonads was sectioned.

C. Acknowledgments

The writer is deeply indebted to Prof. C. E. McClung for having suggested the problem, for providing facilities for work, and for valuable criticisms. He also appreciates the kind interest on the part of the staff of the Department of Zoölogy. He wishes to express his indebtedness to the Marine Biological Laboratory at Woods Hole for facilities for work during the summer of 1927.

OBSERVATIONS

A. Life history

The following account of the life history of Melanoplus differentialis is designed as a background for the observations on the developing gonad. A more detailed account of the early embryonic development will appear later in connection with a study on the origin of the germ cells.

1. Oviposition. Oviposition in Delaware County, Pennsylvania, occurs in the late summer and early fall. Females in large numbers may be observed ovipositing during the middle and latter part of September. Females hatched in the laboratory in June from eggs laid in the early part of October of the preceding year reached maturity the first part of August and oviposited the latter part of August and first part of September. These individuals were kept in cages exposed to the sun during a greater part of the day and probably reached maturity slightly earlier than if allowed the freedom of their natural habitat. For a description of the egg, the oviposition process, and the structure of the egg pod, see Fedorov ('27), McNabb ('28), and Uvarov ('28). The number of eggs deposited in any single pod varies according to the writer's counts from 45 to 151.

2. Development within the egg. For convenience the writer has divided the embryonic development into the prerevolution,[3] early revolution, late revolution, and postrevolution periods as outlined below.

[3] The terms revolution, rotation, and blastokinesis are used somewhat interchangeably in insect embryology. The writer has preferred the term revolution.

a) The prerevolution period.[4] Immediately following deposition embryonic development begins. The early developmental phenomena occur entirely within the caudal cap of the egg. At first the embryo appears as a slightly concavoconvex disc, the concave side closely applied to the yolk (fig. A, *A, bl.*). Later, a silghtly attenuated ribbon-like process may be seen protruding just cephalad to the edge of the caudal cap. The embryo at this stage has two definite regions, the disc-shaped cephalic region and the ribbon-shaped abdominothoracic region (fig. A, *B, c.re.* and *a.t.*).

There seems to be some variation regarding the direction of growth of the abdominothoracic portion of the embryonic rudiment. In the majority of embryos examined this portion grew out over the yolk slightly to the right or left of the ventral median line of the egg. A few embryos were noted, however, in which this growth occurred toward the dorsal side of the egg.

During the latter part of October and the month of November the embryos begin to show considerable regional differentiation. In the head region there are the cephalic lobes, labrum, and antennae; the buccal region is demarcated by the mandibular and maxillar rudiments; the thoracic region, by three pairs of rounded prominences—the rudiments of the walking legs—while the abdominal region continues flattened and ribbon-like (fig. A, *C*).

An examination of embryos during December shows the abdominal region to be definitely segmented. As a result we are able to observe seventeen primitive segments aside from the head, namely, three gnathal, three thoracic, and eleven abdominal segments (fig. A, *D*).

[4] This description of the general course of development through fall, winter, and spring is based upon observations of eggs laid during a period of three weeks in the early fall and kept out of doors in boxes of moist sand. This development is therefore only partially comparable to that which occurs in the natural habitats, where the kind of soil, temperature, moisture, time of deposition, etc., may vary considerably and condition the course of embryonic development accordingly.

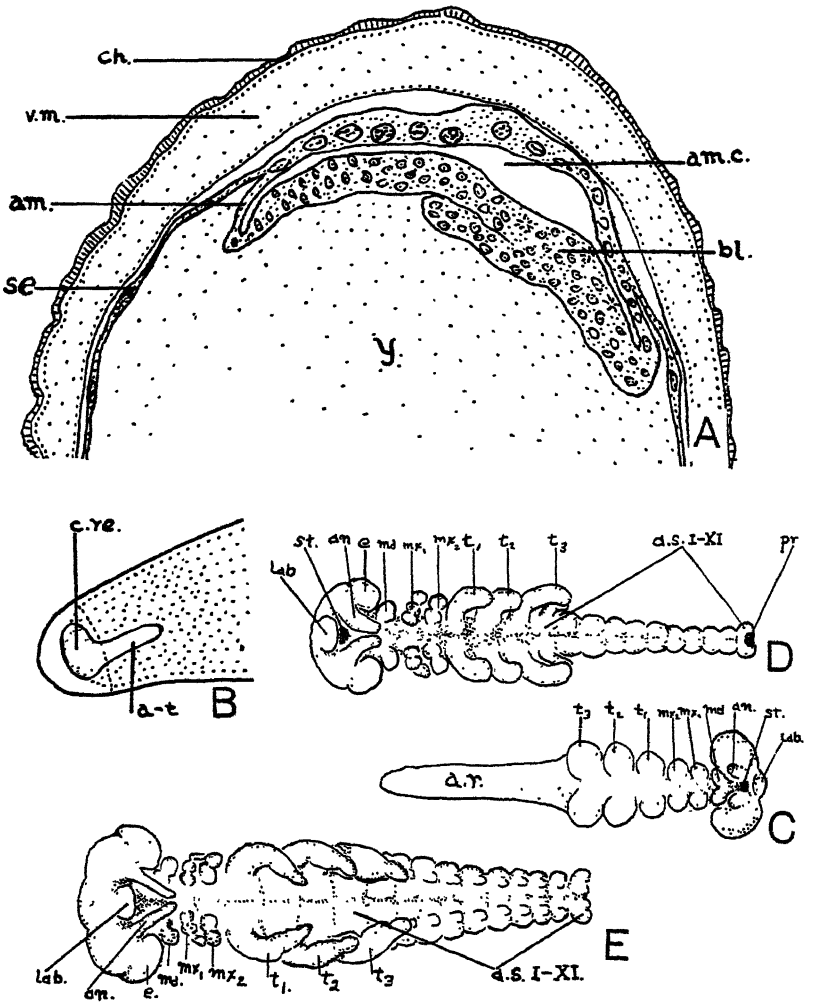

Fig. A A. Section through early blastoderm. B. Early germ band, showing cephalic and abdominothoracic regions. C. Partially segmented germ band. D. Segmented germ band. E. Embryo near end of prerevolution period. *a.r.*, abdominal region; *a.s.*, abdominal segments; *a.t.*, abdominothoracic region; *am.*, amnion; *am.c.*, amnionic cavity; *an.*, antenna; *bl.*, blastoderm; *c.re.*, cephalic region; *ch.*, chorion; *lab.*, labrum; *md.*, mandible rudiment; mx_1, mx_2, maxillae rudiments; *e.*, eye; *pr.*, proctodaeal invagination; *se.*, serosa; *st.*, stomodaeal invagination; *v.m.*, vitelline membrane; t_1, t_2, t_3, walking-leg rudiments; *y.*, yolk.

Present evidence points to a continued slow development through the winter months[5] whenever the temperature is favorable. This development is accelerated during the warmer temperatures of the late winter and early spring. Consequently, in late April or May, depending upon the time of deposition and the temperature averages of the winter months, sectioned embryos demonstrate that the rudiments of the various organs and systems are formed. Embryos at this stage are considerably flattened and have a contracted or huddled appearance. It is then, if the temperature be favorable, that the revolution, or blastokinesis, phenomena occur.

b) The early revolution period. The embryo during its development thus far is situated on the ventral (or slightly ventrolateral) side of the egg. Its head is directed toward the caudal pole and is fastened to that pole by means of the amnion and serosa. A slight movement away from the caudal pole takes place as the yolk is used up. When development has proceeded as outlined above, other movements are initiated. Active contraction phenomena are initiated in the tissues of the embryo. This is followed by the splitting of the fused amnion and serosa along the ventromedian line of the embryo, the serosal attachment to the caudal pole is disrupted, and the serosa begins to retreat toward the cephalic pole of the egg. Subsequently, the embryo moves anteriorly around

[5] Wheeler ('93) mentions a hibernation period or "period of quiescence due to cold weather" in the case of the embryo of Gonocephalus (Xiphidium), and Carothers ('23), in a paper devoted to developmental phenomena and taxonomic features in the Acrididae, on page 12 makes the statement, "Most species normally hibernate in the egg. They develop to a certain point and stop until the necessary conditions for further development have been met." In M. differentialis the writer has not observed this quiescent or hibernation period under out-of-door conditions. On the other hand, if egg pods are incubated at 25°C. from the time of deposition, the ensuing development is relatively rapid up to near the end of the prerevolution period. In the case of the majority of embryos in any single egg pod development ceases at this temperature at this time and a quiescent or hibernation period is experienced. A few embryos in each pod may not undergo this quiescence even at higher temperatures, continuing in development. These phenomena will be described more completely in a later paper.

the attenuate pole and up the dorsal, dorsolateral, or in some cases around to the lateral side of the egg.[6] The exact course followed is seemingly dependent upon the position of the embryo previous to the initiation of the movement. When the embryo becomes straightened out in the latter position, it immediately begins a lateral rotation[7] either to the right or left, depending upon its position, toward the ventral side of the egg. It reaches the latter situation in a relatively brief time. The head of the embryo is now directed toward the cephalic end of the egg (fig. B, *F*).

The principal changes in the external features of the embryo during this period appear to be mainly one of contraction along the cephalocaudal axis and a spreading of the lateral margins as they begin to encompass the yolk (fig. 15).

It is interesting, as a comparison to the foregoing, to recall the statement of Wheeler ('93, p. 72) regarding the development of M. femur-rubrum:

> The germ-band is formed very near the caudal pole of the egg, but still on the convex ventral surface. During the formation of the envelopes the posterior end of the body grows around the pole onto the dorsal surface while its head remains fixed at the pole. It is not until the germ band has reached a stage corresponding to stage F. in Xiphidium (i.e. complete segmentation), that its head leaves the pole and the whole body moves upward on the dorsal surface. It soon

[6] As the reader will observe, there are two sets of movements involved in the revolution phenomena, namely, the movements of the embryonic membranes and the movements of the embryo. The membrane movements consist in the rupture of the serosal attachment to the caudal egg pole, the splitting of the fused amnion and serosa, the contraction of the serosa, and the eversion of the amniotic cavity. The eversion of the latter is apparently brought about by the splitting in the area of fusion between the amnion and serosa and the subsequent serosal contraction. The movements of the embryo may be described as directional and intrinsic. The directional movements consist in the passage of the embryo around the caudal pole, the laterad manoeuvering to the ventral side of the egg, and the cephalic movement toward the cephalic egg pole. The intrinsic movements occur within the body of the embryo. Aside from intermittent jerking and twisting movements, regulated contraction waves may be observed extending cephalad along the lateral margins to the head region. These contraction waves are feeble at first, but become more pronounced as the revolution process continues. They probably represent heart-rudiment contractions.

[7] In some instances this lateral rotation does not occur.

comes to a standstill and passes the winter in this inverted position. In the spring it moves back around the lower pole and . . . . proceeds to lengthen and envelop the yolk till it reaches the cephalic pole.

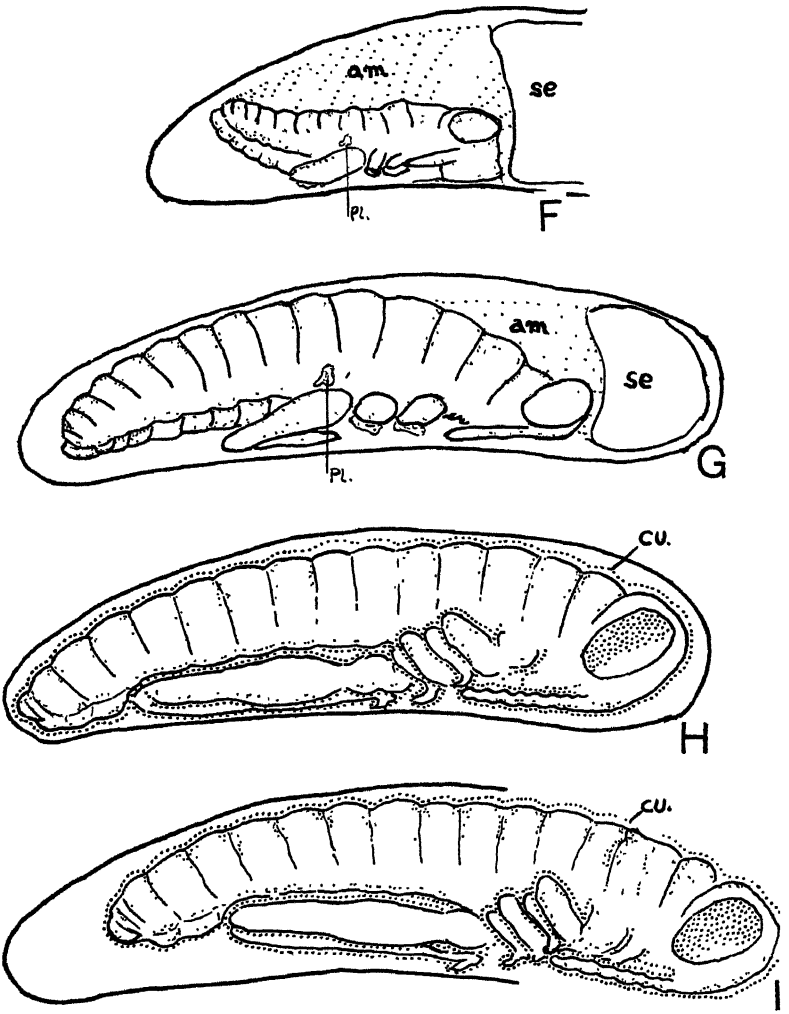

Fig. B F. Embryo at beginning of late-revolution period; G. Embryo toward end of late-revolution period; H. Embryo at middle of postrevolution period. I. Pronymph or vermiform larva. *am.*, amnion; *cu.*, cuticle (represented very diagrammatically); *pl.*, pluropodium, first abdominal appendage; *se.*, serosa.

c) The late revolution period. The embryo has now attained its final orientation in the egg (fig. B, *F*) and begins a period of development during which the yolk is engulfed and the embryonic envelopes consumed. Figure 16 shows the relationship of the embryonic membranes to the embryo and the yolk just after the early revolution period is completed. It is to be noted that the contracting serosa has drawn the amnion back over the yolk. The embryo envelops the yolk ventrolaterally and caudally. The amnion, attached to the up-growing ectoderm of the lateral margins of the embryo and anteriorly to the serosa, covers the yolk dorsad of the embryo, while the anterior portion of the yolk is enclosed by the serosa. As the embryo grows cephalad in the egg, the lateral margins in their upgrowth fuse in the median dorsal line, from the posterior to the anterior, part of the amnion (fig. 20, *am*) being utilized during this fusion process. Gradually the yolk is enclosed, until finally the head of the embryo approaches the cephalic extremity of the egg, where the remaining yolk and the remaining portions of the amnion and serosa are taken into the embryo in the median line between the eyes and the upper part of the front of the head (fig. 17, *um.*, also fig. B, *G* and *H*).

During this process, and paralleling the growth of the lateral ectodermal margins up over the yolk, the enteron rudiment grows up around the latter, ultimately fusing in the middorsal line. Simultaneously other structures, such as the fat bodies, rudiments of the heart, muscles, and gonads, are carried dorsad. In the ventral region there is a marked development of the nerve cord. The commissures are established between the ganglia, and a gradual fusion of the various ganglia occurs. As is noted below, the sexes are differentiated now.

The length of this period varies with the temperature as do the other developmental stages. At a room temperature varying between 18°C. and 25°C. the yolk and membranes were enclosed in the case of the embryos in two egg pods under observation within four days. Under outside condi-

tions existing during the latter part of May, 1927, the majority of embryos in a pod, of which representative eggs fixed on the 22nd of May showed that the first phase of the revolution movements were being completed on that date, had completed the late revolution phenomena by the 29th of May.

d) The postrevolution period. Immediately after the circumcrescence of the yolk and the consumption of the embryonic envelopes, the embryo secretes a chitinous cuticle around itself, including each of the appendages (fig. B, *H* and *I*). This appears similar in section and staining to the chitinous cuticles secreted successively during postembryonic development. It retains this cuticle until the time of hatching. For a description of the shedding of this cuticle, see Hagen ('17) and Uvarov ('28).

Changes during this time involve the progressive development of the various organs and systems to such an extent as to permit the developing animal to cope with conditions without the egg membranes. Its duration is variable with temperature. At a temperature of 25°C. it is approximately eleven days. The embryos of a pod of eggs in which representative eggs showed that the revolution phenomena were being completed by the 1st of June, 1927, under outside conditions, hatched June 20th.

3. Hatching and the pronymph or vermiform larva. The postembryonic period of development is initiated when the larva emerges from the egg. As the hatching process has been detailed by Uvarov ('28) and others, it will not be repeated here. It is sufficient to say that the larva, by a series of worm-like movements and pulsations of the cervical ampulla, ruptures the egg membranes and ultimately reaches the surface of the soil. During this procedure, or shortly after it reaches the surface, it sheds the chitinous cuticle referred to above.[8]

[8] Temperature appears to have considerable influence on the emergence of the larvae. The writer has kept eggs due to hatch at a cool temperature for a period of five days. Eggs from that particular pod brought into a room, where the temperature averaged 22° to 25°C. from day to day, hatched a few hours after they were brought into the warm room. At the end of the five days the remaining

The larva, during the period which intervenes between the emergence from the egg and this first ecdysis or shedding of the cuticle, has been termed the vermiform larva, and the first molt has been designated as the intermediate molt (Uvarov, '28). In the Odonata this larva has been called the pronymph (Balfour-Browne, '09) and similarly in the Orthoptera and Odonata (Walker, '20).

4. The instars. The external postembryonic development of the Acrididae, in general, has been treated in the literature.[9] Internally, marked changes occur in the reproductive systems which at the time of hatching are rudimentary. The development of the male gonad is noted below.

This species of Melanoplus appears to be somewhat unique,[10] in that it experiences seven ecdyses before the adult condition is reached. There are subsequently six periods (instars) of postembryonic development.

The duration of the instars varies with temperature and probably with the kind of food. For the effect of temperature on insect development the reader is referred to the papers of Bliss ('26), Bodine ('25), and Ludwig ('28). In this connection see also Bodenheimer ('27) and Calvert ('29).

5. The adult. In the laboratory the writer has observed copulation and oviposition occuring three weeks after the last

eggs of the pod were brought into the warm room and they hatched within six hours. Light does not appear to influence the hatching process. But according to Uvarov ('28), the observations of La Baume indicate that larvae which emerge in darkness fail to experience the intermediate or pronymphal molt unless they are brought into the presence of light. Just how long this molt may be suppressed in this manner is not indicated.

[9] For example, see Criddle ('26) and Uvarov ('28).

[10] A considerable number of hoppers already studied have shown a postembryonic development of five instars. There seems to be some variation, however, as is shown by the following taken from Uvarov ('28). "Bie-Bienko ('28)—Four larval stages in several small species in Siberia. Dovnar-Zapolsky ('26)—Pesotettix giornae has six larval stages. Plotnikov ('26)—Dociostaurus kraussi, the males pass through four larval stages and the females through five stages. Coleman ('11)—Hieroglyphus banian, the males reach the adult stage after six stages, while the females pass through seven stages. Coleman and Kannan ('11)—Colemania sphenarioides may show either five or six stages irrespective of sex." Criddle ('26) reports six instars for Pardalophora apiculata and also six for Xanthippus latifasciatus.

ecdysis. Individuals have been known to live under laboratory conditions for a period of forty-five days or longer after the adult molt occurred, and out of doors during the summer adult animals have been kept for a slightly longer period than two months.

B. Sex differentiation

1. The external genitalia. At the beginning of the revolution phenomena two groups of embryos may be noted. In one the tenth pair of abdominal appendages[11] is well developed, in the other it is vestigial (figs. 1 and 2, *X*). This is the first indication of a distinction between the two sexes. The group with the tenth pair of appendages well developed are males, while the other group are females.

During the late revolution period the tenth pair of appendages in the male moves mesiad and cephalad. A little later they appear as rounded structures closely associated with the inner basal portions of the ninth pair of appendages, which have also moved mesiad (fig. 4, *IX*, *X*). At the close of the late revolution period the ninth pair of appendages and the ninth sternite have formed one piece, the rudiment of the genital plate (fig. 6, *gp.*, and figs. 8, 10, 12, and 14). The tenth pair of appendages in their cephalad migration disappear from view. Present observations on embryos sectioned at this stage point to the latter pair of appendages as forming, in part at least, the rudiments of the ejaculatory duct and penis.

The eleventh pair of appendages continue their differentiation into the cerci. The other abdominal appendages disappear.

In the developing female the tenth pair disappears during the early part of the late revolution period. The ninth pair together with the eighth and seventh pairs moves mesiad. As this period terminates the ninth pair is attached near the median line to the posterior border of the ninth sternite. The

[11] The writer has preferred to regard these rudiments as abdominal appendages. However, the reader is referred to Korschelt-Heider ('99), pp. 295–300.

eighth pair, which has undergone a relative reduction in size, appears as two knob-like structures attached to the posterior edge of the eighth abdominal sternite. The seventh pair of appendages persists to this time and has a similar relationship to the seventh sternite. This pair later disappears. The eighth and ninth pairs become ovipositors in the adult female, (figs. 1, 3, 5, 7, 11, and 13). The fate of the other appendages is similar to that in the male.[12]

2. The genital rudiments. At the time of the completion of the revolution arcs, the genital rudiments in both sexes are similar. It is not until the latter part of the late revolution period and the early part of the postrevolution period that a distinct sexual difference in the rudiments can be noted. This difference is described below.

C. The development of the gonad

1. The early genital rudiments. The following description applies to the genital rudiments at the beginning of the late revolution period, immediately after the revolution arcs have been completed.

At this time each rudiment consists of a lateral cord of cells extending from the first to the eighth abdominal segments.[13] Each cord is attached dorsad at the junction of the enteron and dorsal diaphragm rudiments by a thin epithelial mem-

[12] The course of the development of the external genitalia in Gonocephalus (Xiphidium) ensiferus, as observed by Wheeler ('93), corresponds closely with that in M. differentialis. The writer, however, cannot concur in the belief regarding the female "that the tenth pair of embryonic appendages persists and moves forward to join the ninth pair," as the evidence in M. differentialis suggests a complete disappearance of the tenth pair.

[13] Heymons ('91), in Phyllodromia germanica, found the genital rudiments to extend from the second through the seventh abdominal segments. Wheeler ('93), in Gonocephalus (Xiphidium), observed germ cells distributed from the first to the sixth abdominal segments and in some instances as "far back as the tenth segment." The exact determination of the limits, cephalad and caudad, is made difficult by the fact that there is a region at each end of the rudiment where the germ cells are few in number, the rudiments consisting mainly of epithelial cells. This varies somewhat in different embryos. The interpretation by different observers regarding the exact limits of the rudiment in the same species may therefore vary slightly. (See Wheeler, p. 123.)

brane which may be designated as the 'terminal filament,'[14] ventrad each is in intimate association with the rudiment of the fat-body. In length each cord averages 0.7 to 0.75 mm. In some regions the dorsoventral diameter, exclusive of the terminal filament, approaches 83 μ, while the lateral diameter approximates 33 μ. In other regions the genital cord is smaller, having a dorsoventral diameter of 70 μ and a lateral diameter of 19 μ. The number of cells visible, therefore, in any series of transverse sections will vary.

In transverse section each genital rudiment is spindle-shaped and presents the following structural features:

a) The terminal filament noted above.

b) The dorsal cell mass. This term is applied to the group of cells situated in the tapering portion just below the terminal filament (fig. 23, *d.c.m.*). These cells are epithelial in character, with relatively large oval-shaped deeply staining nuclei. The cytoplasm is small in amount.

c) The central cell mass. This designation is used for the cells of the bulging portion of the rudiment. It is composed of germ cells between which are interspersed mesodermal cells with nuclei of various shapes. These interspersed cells are similar to the cells of the dorsal cell mass.

The germ cells average 12 μ to 14 μ in diameter. The nuclei are spherical or slightly invaginated and fill the cells considerably (fig. 23, *p.g.c.*). The contents of the nuclei stain faintly, with the exception of two or three irregular-shaped, subspherical, or slightly elongated masses of chromatin which stain deeply. At times certain of these deeply staining masses have a form much like the accessory chromosome of the maturing sperm cell. The homogeneous cytoplasm is small in amount, and in some cells appears as a very narrow rim around the nuclei. In the cells where the nuclei are slightly invaginated the cytoplasm tends to be concentrated at the side

[14] The writer has applied the term 'terminal filament' to this membrane, but it must be observed that it is not identical with the term used, for example, by Heymons ('91) in reference to a similarly situated plate of cells in Phyllodromia. Heymons uses the designation 'Endfadenplatte' to also include what the writer describes as the dorsal cell mass.

of the cell where the invagination occurs. There is no evidence of included yolk material in the cytoplasm. The germ cells show no mitotic activity. The number observable in any one section along the cord varies from one to eleven cells; but at the ends of the cord, in the first and eighth segments, they are considerably reduced in number and may not be present at all in several sections before their final disappearance. Immediately cephalad and caudad to the region containing the germ cells the rudiment is composed solely of epithelial elements.

d) The ventral cell strand. Ventrad, just below the central mass of cells, groups of two, three, or more cells may be observed. They represent the beginning of the 'ventral cell strand'[15] (figs. 23, 24, and 25, *v.c.s.*). This becomes a well-developed structure in the postrevolution period (figs. 28, 29, *v.c.s.*) and is destined to play an important rôle in the development of the efferent genital duct. The cells are similar to the epithelial elements of the dorsal cell mass.

e) The outer limiting membrane. This is a thin, delicate epithelial membrane closely applied to the mesial and lateral surfaces of the genital rudiment. It extends from the fat-body to the terminal filament with which it merges. It may vary from 0.5 μ to 4 μ in thickness. Distributed in this membrane are a few elliptically shaped nuclei (fig. 23, *o.l.m.*).

f) Isolated germ-cell groups. Scattered in the fat-body ventrad to the genital rudiment, and associated with the cells of the enteron rudiment, there are groups of one to three isolated germ cells. Present evidence points to their ultimate degeneration.

2. The genital rudiment during the latter part of the revolution period. As the upgrowths of the lateral margins and mesodermic structures continue they ultimately meet in the middorsal line, as shown semidiagrammatically in figures 20, 21, 22. The rudiments of the dorsal diaphragm, *d.d.*, and the splanchnopleure, *spn.*, to which is attached the terminal filament, *t.f.*, merge at point *b* (figs. 20 and 21). During this

[15] See Korschelt and Heider ('99), p. 347.

merging process, *d.d.* and *d.d.* come together to complete the dorsal diaphragm (fig. 21); *t.f.* and *t.f.* unite as the connecting filament of the future gonad (compare figs. 21 and 22); while *spn.* and *spn.* join, enclosing the yolk and completing the dorsal wall of the midgut (figs. 21 and 22). The gonadal rudiments (genital ridges), *g.r.*, now extend in the coelomic space between the heart and the splanchnopleure of the midgut united by the connecting filament, *c.f.*, and joined to the fat-bodies, *f* and *f*, laterally. When this final relationship is established, the yolk and the embryonic envelopes are engulfed. Before considering the structure of the rudiments at this stage, let us briefly observe their condition during the late revolution phenomenon when the relationships of the above structures are as shown in figure 20.

The genital cords change considerably during the late revolution period. They elongate to the extent that their length approximates 1.08 mm. each, extending from the cephalic part of the eighth abdominal segment into the third segment. The expansion of the abdominal segments, as the embryo grows dorsad and cephalad around the yolk, accounts for the shift of the cephalic limit of the cords from the first to the third segments. During this increase in the length of the rudiment the germ cells remain constant in number, but they are distributed longitudinally along the cord, as is shown by the decrease in the number of germ cells observable in transverse sections cut along the rudiments with the exception of the caudal portions, which lie in the seventh and eighth segments. In the latter segments two to five germ cells may be found per section. Cephalad to these segments there is seldom more than two or three germ cells per section and it is not uncommon to find sections devoid of germ cells. Aside from the distribution of the germ cells along the extended genital rudiment, the rudiment itself is undergoing important changes. In the first place, the ventral cell strand (fig. 22, *v.c.s.*) is now a prominent structure. Secondly, during the latter part of the yolk engulfment process an important change occurs in the male rudiment, which is not observable in the female.

The cells of the dorsal cell mass of the former begin to move back among the cells of the central mass (fig. 25, *d.c.m.*). In the female this structure remains as a conspicuous mass of epithelial cells (fig. 27, *d.c.m.*). At the beginning of the post-revolution period this difference in the two rudiments is readily detected[16] (compare figs. 26 and 27, *d.c.m.*).

3. The genital rudiment at the beginning of the postrevolution period. Shortly after the yolk and envelopes are engulfed, the germ cells in the male genital rudiment begin mitotic activity. Sections of the gland at this time show many early and late prophase stages (fig. 26). The obliteration of the dorsal cell mass by the movement of its cells into the germ-cell portion of the rudiment causes each to appear oval in shape, with the ventral cell strand extending laterad and slightly ventrad in direction.

The rudiments extend from the third into the seventh abdominal segments. There is a slight convergence from the cephalic to caudal extremities, and the connecting filament (fig. 22, *c.f.*) varies subsequently in length.

It appears that an actual contraction of the rudiments has been effected when a comparison is made of their present extent with that of the late revolution period.[17] This is shown by the length of each cord, which now approximates 0.9 mm., and also by the fact that their caudal extremities do not extend as a rule beyond the seventh abdominal segment, being confined within this segment.

The caudal portions of the genital cords, particularly in the seventh abdominal segment, show more germ cells per

[16] The time of the appearance of the distinguishing sex features in the genital rudiment in M. differentialis closely parallels their appearance in Phyllodromia germanica. Concerning the latter, Heymons ('91) writes: "Sobald die genitalanlagen ihren difinitiven Plaz im Korper einnehmen, trifft man die ersten sexuellen Unterschiede an." The details of the differentiation are not the same, however. The distinguishing feature which Heymons observed were four accumulations of genital cells in the male rudiment. In M. differentialis these accumulations, or segregations, of genital elements into definite groups (the cell nests) do not occur until the latter part of the postrevolution period.

[17] See Nelson ('15) regarding a contraction of the rudiments of the sex glands in the honey bee and the mason bee, pp. 216, 217.

section than the more cephalic regions. A probable explanation of the concentration of germ cells in this region may be found in the activities of the growing embryo as it extends cephalad in the egg during yolk circumcrescence. The genital cords are carried along in this process with the general mass of tissues, so that the extent of the cords is greater at the end of the yolk-enveloping process than at the beginning. And as the germ cells are not dividing during this period, the number in any one section is reduced. However, the caudal portion of the embryo is relatively fixed, so that the tissues in this region will be disturbed less during this growth than will tissues located more cephalad. It follows that the germ cells in the caudal portions of the genital rudiments will tend to remain in the position that they had previous to the engulfment process. Consequently, successive transverse sections in this particular area give counts of two to four cells per section, while sections elsewhere in the genital rudiments generally show one or two germ cells, and sections without any are not uncommon.

Cephalad, each genital ridge continues for a short distance as a mass of epithelial cells, which, together with the connecting filament, form a flattened membrane, joined to the fat-bodies laterally, and extending cephalad for a short distance.

4. The genital rudiments at the middle of the postrevolution period. Mitotic activity and differentiation in the developing gonads continue throughout the postrevolution period. At the middle of this period a transverse section of the embryo through the region of the genital rudiments presents the following structural features in regard to the rudiments and the surrounding tissues.

a. The average width of each rudiment is 0.04 mm. and the dorsoventral diameter, 0.03 mm. Each is roughly oval in shape (fig. 28).

b. The outer limiting membrane (fig. 28, *o.l.m.*) extends around the gonadal rudiment, enveloping the ventral cell strand as well as the rudiment proper. It contains numerous oval-shaped or flattened nuclei undergoing mitoses. The por-

tion surrounding the ventral cell strand may contain a few small nuclei (fig. 28, *v.c.s., o.l.m.*).

c. The indifferent mesodermal cells are differentiating into connective-tissue elements and form partial or complete partitions among the germ cells, isolating them into groups of one or more (figs. 28 and 29). The region of the gonadal rudiment at the juncture of the ventral cell strand appears to be an area from which undifferentiated cells probably move up among the germ cells during this segregating process. As will be observed later, this area becomes very active in the formation of the follicles and the gonadal portion of the vas deferens. Because of this activity and importance in the development of the testis, we shall designate it as the germinal center (fig. 28, *g.ce.*).

d. The ventral cell strand is now a conspicuous structure. It is attached to the developing genital gland in the midventral region, and extends in a ventrolateral direction for a distance averaging 0.027 mm. It has moved mesiad from the lateral position which it occupied at the beginning of this period (compare figs. 22, 26, and 28, *v.c.s.*). In certain areas the distal portion is slightly enlarged (fig. 29, *v.c.s.*).

e. The fat-bodies extend laterad and dorsad in the coelom and envelop the genital ridges dorsally.

f. The dorsal diaphragm and genital rudiments are almost contiguous above, while the developing enteron musculature presses close to them ventrally, due to the engulfed yolk within the midgut.

5. The genital rudiments at the time of hatching. The activity within the developing genital glands during the post-revolution period results in the formation of groups of germ cells separated by connective-tissue cells and other partially differentiated or non-differentiated mesodermal cells. In an average transverse section of the genital rudiment at the time of hatching three or four such groups may be detected, either in a state of complete or partial isolation (fig. 31). This inner framework between the groups of germ cells is distinct from the outer limiting membrane, which forms the

outer capsule of the developing gonad. However, the cells and processes forming the peripheral portion of the internal framework are in intimate contact with this membrane. At the end of the postrevolution period we thus find the genital rudiments in a state of transition from a condition of partial organization to one of organization and differentiation. Before continuing the observation of this process of differentiation, we will pause to delineate the genital rudiments as a whole and observe their relationships with other body structures.

The rudiments of the gonads at the time of hatching are in the form of two elongated, slightly separated cords immediately ventral to the heart, and extend from the caudal half of the third to the cephalic half of the sixth abdominal segments. Each cord approximates 0.65 mm. in length, averages 0.06 mm. in width, and in its widest diameter dorsoventrally measures approximately 0.03 mm. Its shape is oval in cross-section. The outside contour presents a series of slight bulgings. Each of these bulgings represents a differentiating follicle rudiment. The ventral cell strand is joined to the ventral mesial edge of each cord near the point of attachment of the connecting filament.

The fat-body, which surrounds the genital cords, appears to be delicately attached to the dorsal diaphragm. The entire mass of fat-body and genital rudiments is apparently suspended in the coelomic cavity from the dorsal diaphragm. A narrow coelomic space intervenes between this suspended mass and the stomach (midgut), and in this space malpighian tubules may be observed. The tracheal sacs do not penetrate into the dorsal coelomic region in the vicinity of the rudiments at this time.

While the foregoing description is intended as typical for conditions within and without the gonadal rudiments at the time of hatching, it must be called to mind that the time of hatching is a relative period, dependent upon external as well as internal factors. Hatching evidently does not demarcate a particular, but an approximate state of development, as

some gonads are slightly more advanced than others. In some there are considerable numbers of apical cells already differentiated; in others, there are only a few, if any.

6. Apical cell formation. Starting with the germ-cell group, or cell nest, which may consist of from one to four or five germ cells surrounded by more or less differentiated connective-tissue cells, we observe the following. In the case of cell nests consisting of one or two germ cells there occurs a division of the cells. When the number has increased to approximately four or five, an indifferent mesodermal cell may be seen pushing in among the germ cells. This occurs from the periphery of the gonad. That is, a cell which has been situated in close proximity to, or in actual contact with, the outer limiting membrane, insinuates itself among the germ cells of the nest. It moves inward, gradually extending its processes among the germ cells, and ultimately joins these processes with the processes of the surrounding cells of the nest. During this procedure the germ cells continue to divide. When it is completed, a cross-section of such a group of cells through the central cell presents from five to seven germ cells surrounding the central cell. Enveloping the entire group is a surrounding capsule of connective-tissue cells. The processes of these capsular cells (even parts of the cell bodies and nuclei) protrude among the germ cells. (For steps in this process, see figs. 29, 31 to 36, *ap.*) This results in the establishment of an ordered framework of connective-tissue cells, a central cell, and capsular cells whose processes form a close union. Between these processes the germ cells lie. The central cell we may designate as the 'apical cell,' the germ cells as 'primary spermatogonia,' and the surrounding connective-tissue cells as the capsular cells (fig. 37, *ca.c., ap., p.sp.*). We shall refer hereinafter to the entire complex of apical, germ, and capsular cells as the apical complex.

As already intimated, all of the apical complexes are not established at once. The actual period of time consumed in their formation is uncertain. Cells in the process of differ-

entiating into apical cells have been observed in rudiments fixed during the late postrevolution period. Apical cell differentiation has also been observed in several instances in those fixed during the latter part of the first instar. However, material fixed at the time of hatching and shortly after generally shows large numbers of cells in the process of becoming apical cells. Probably it would be the more accurate to say that apical cell formation begins shortly before hatching, is greatly accelerated at the time of hatching and for a brief time thereafter when the majority of them are formed, a few remaining apical cells being differentiated toward the middle and latter part of the first instar.

7. The formation of the follicle rudiment. During the establishment of the above relationship of connective cells and germ cells, the uninterrupted increase in the number of germ cells causes the general mass of the differentiating follicle rudiment to bulge, carrying the outer limiting membrane of the gonadal rudiment with it (fig. 36). As this is occurring, undifferentiated cells from the germinal center (fig. 36, *g.ce.*) begin to grow up against the apical complex in funnel-like formation, the cells forming the mouth of the funnel coming in contact with the capsular cells (figs. 36 and 37, *f.d.*). This produces a bouquet effect. Simultaneous with these events the outer limiting membrane begins to grow in toward the germinal center in the regions between the developing follicles (fig. 36, *ingr.*). This ingrowth continues until finally, at the beginning of the second instar, the outer limiting membrane may be seen partially invading the germinal center (fig. 37, *ingr.*). By its invasion of the latter the rudiments of the follicle ducts are formed. The fundaments of the future follicle may now be said to be established.

8. The developing gonads at the time of the second instar. The gonads, at the beginning of the second instar, are situated, as previously indicated at the beginning of the first instar, with the exception that they now extend from the caudal part of the third, into the cephalic part of the fifth abdominal segments. This relative shift has apparently been

brought about by the growth of the animal as a whole with a consequent cephalocaudal elongation of the abdominal segments. In transverse section each developing gonad appears fan-shaped, the growing follicles spreading out from the germinal center, dorsad and laterad in an arc which tends to approach 180°. Parts of three, four, or five follicles appear in each section. The fat-body extends in between the follicles in the form of a delicate network, constructing thereby a fatty sheath around each follicle and filling the interfollicular spaces. Immediately below the connecting filament is an elongated portion of the fat-body which may fill, together with the malpighian tubules, the entire coelomic space between the gonads and the midgut. The developing genital glands are virtually pillowed on all sides by the fat-body. The latter, in fact, tends to fill all free coelomic space in this region of the animal.

The length of each gonad averages 0.85 mm., their combined width 0.30 mm., and the follicles extend from the germinal center at a distance of 0.06 to 0.08 mm.

As shown in figure 38, secondary spermatogonia (cell *a*) and spermatocyst formation (*y.cyst.*) is now underway, and continues throughout the second instar. At the end of this period, the follicle appears club-shaped, the distal end being enlarged, whereas the more proximal portions taper in a gradual manner to the region of the follicle duct (fig. 39). The following features may be distinguished in respect to the follicle at the end of the second instar:

a) The follicular covering membrane. This membrane envelops the follicle and its duct externally (fig. 39, *o.l.m.*). It arises from the outer limiting membrane of the gonadal rudiment as previously described. It responds positively to collagen stains. Numerous elliptical-shaped nuclei are present.

b) The apical complex. A transverse section through the apical cell shows eight or ten primary spermatogonia in contact with the apical cell (fig. 39). Some of the germ cells are closer to the apical cell than are others. The entire complex

appears as an irregular, somewhat spherical, mass of cells with some of the germ cells being crowded out from the circle of the other germ cells.

c) The intrafollicular connective-tissue cells. The apical complex is in actual contact with the outer covering membrane of the follicle distally and partly on its lateral surfaces (fig. 39). In a large number of follicles the complex is displaced to one side, i.e., it is eccentrically situated in the distal end of the follicle. The intervening space between the complex and the outer membrane is filled by young cysts and connective-tissue cells.

Other groups of connective-tissue cells may be seen distributed among the young cysts situated nearer the follicle ducts. These are in contact, by means of their processes, with the connective-tissue elements along the periphery of the developing follicle, contiguous with the outer covering membrane. There is also a central core[18] of cells distributed in the central portion of the follicle. This core extends proximally from the apical complex for some distance (fig. 39, *c.c.*). In the more mature follicle it has somewhat the same supporting relationship to the young cysts that the apical cell has to the primary spermatogonia (fig. 45).

These connective-tissue cells form a continuous growing intrafollicular framework. Some of its constituent cells may have arisen from mitotic activity of the capsular cells of the apical complex, but the greater number have undoubtedly taken their origin from cells derived from the germinal center during the differentiation of the follicle rudiment (compare figs. 37, 38, and 39).

During the second instar the principal change in the dimensions of the developing gonad results from the elongation of the follicles. Near the close of the instar the follicles average 0.12 mm. in length, with a direction of growth tending dorsad. In the case of the more peripheral follicles the direction of

[18] Davis ('08) applies the name 'longitudinal rachis' to this central core. Sutton ('00) seems to apply the designation 'longitudinal rachis' to the developing gonadal vas deferens.

growth is necessarily dorsolateral. The combined width of the gonads averages 0.35 to 0.40 and their length reaches 1.035 mm.

9. The differentiation of the ventral cell strand and germinal center. The ventral cell strand, as viewed in transverse section, appears first as a group of two or three epithelial elements associated at the ventral portion of the early genital rudiment (fig. 23, *v.c.s.*). This later changes to a triangular-shaped structure (fig. 24) and at the close of the yolk-engulfing process its form is that of an elongated cell strand with its constituent epithelial elements arranged in row-like formation (figs. 22, 25, 26). Its covering membrane is the outer limiting membrane of the gonadal rudiment which continues into the fat-body at the distal end of the strand.

During the postrevolution period, as previously indicated, the ventral cell strand in the male shifts from a lateroventral position to one that is mesial and ventral in relation to the main body of the rudiment (compare figs. 26, 28, 29, 31, and 32). As this change is correlated with developmental rearrangements within the genital ridge as a whole, the germinal center likewise moves mesiad. At the beginning of the second instar, therefore, the relative positions of these two structures are as shown in figures 36 and 37.

At this time the germinal center (figs. 36 and 37, *g.ce.*) appears as an irregularly rounded body approximating 0.016 mm. in diameter; the ventral cell strand is elongate in form, with its distal portion tapering to a point, continuing into the fat-body as a flattened membrane, the lateral filament. The length of the ventral cell strand from the germinal center to the lateral filament is variable, roughly averaging 0.04 mm., while its width in the proximal portions reaches 0.008 mm. The enveloping membrane surrounding these two structures is devoid of nuclei around the ventral cell strand, but there are a few scattered oval nuclei in the membrane covering the germinal center, especially in the portions nearest the follicle ducts. This membrane responds to the acid fuchsin in the stain of v. Gieson with a brilliant red coloration. Because

of this it appears quite distinct from the portions of the membrane which envelop the follicles, the latter responding positively, but with a color which is much less intense. This is probably due to the diminished thickness of the latter and also to the fact that it is enlarging rapidly in response to the general growth of the follicle as a whole.

It is interesting to observe in this connection that the cell body and processes of the apical cell, as well as the processes of the included connecting cells of the follicle, respond to the fuchsin of this stain with a faint pink coloration, whereas the cytosomes of the germ cells appear faintly yellow.[19]

There is a feature of the ventral cell strand and germinal center which at this stage of development forecasts their ultimate situation in the fully developed gonad. In certain limited regions, the outer enveloping membrane tends to pinch in and separate the cells of the ventral cell strand from those of the germinal center (fig. 41, *ingr.*). Elsewhere the cells of the germinal center and ventral cell strand are continuous (fig. 40). The importance of this ingrowth in the ultimately differentiated structure is noted below.

The general morphological relationships of these two structures to each other are retained, as here outlined, throughout the remainder of their development. Aside from changes in shape and size, there are two phenomena in regard to their cellular make-up which are important.

Internally, there is an increase in the number of cells. Some of these cells become arranged during the instars to form a definite epithelial layer of a simple columnar variety lining the inner surface of the enveloping connective-tissue membrane. These cells retain their contiguity with the remaining cellular elements until the latter part of the sixth instar.

[19] Lewis and Robertson ('16), describing the apical cell in Chorthippus curtipennis, state that "The somatic cells, which form the wall of the follicle, have abundant neutral red granules and these stain with neutral red in much the same manner as did those of the apical cell. This striking resemblance of the apical cell to the somatic cells in contrast to the germ cells suggests the possibility that the apical cell may be more closely related to the somatic cells than to the germ cells."

There is a marked difference in the number of cell constituents of the ventral cell strand and the germinal center. In the former there are sufficient cells formed to pave the enveloping membrane, whereas in the latter there is a considerable mass of epithelial elements which takes no part in the formation of the inner lining of this membrane. In certain regions there is a slight concentration of excess cellular elements in the proximal portions of the ventral cell strand, but this may be due to a crowding in of cells from the proliferation occurring in the germinal center.

Externally, a contemporaneous change occurs in the structure of the enveloping membrane. Within the membrane surrounding the germinal center, the cells already present divide and some of them move toward that portion of the membrane surrounding the ventral cell strand. There is also a possibility that some of the cells within the ventral cell strand, contiguous with the enveloping membrane, may move into the membrane. But this is not clearly demonstrable from the preparations which the writer has at present. Whatever the exact process, the membrane surrounding the germinal center and ventral cell strand contains a few scattered cells at the end of the fifth instar. These cells are the loci of new structural specializations.

Layers of flattened fibers become associated about the nuclei of these cells and extend parallel to the longitudinal axes of the germinal center and ventral cell strand. In transverse section they appear as oval or elliptical-shaped bundles of fibers, and wherever nuclei are present, the fibers appear to radiate at right angles to the nuclear surface. The bundles are larger and more conspicuous in that portion of the membrane surrounding the germinal center, particularly in the regions where the follicle ducts join the center (fig. 44, *el.*). To the stains of v. Gieson and Mallory these fiber bundles respond with a yellowish coloration, and to orcein with a color which is deep brown.

As the adult molt approaches, conspicuous internal changes in the cellular relationships of the germinal center and ven-

tral cell strand are noticeable. The cells lining the inner surface of the enveloping membrane separate from the more central mass of cells of the germinal center. Likewise, the apposed layers of the ventral cell strand move apart (figs. 42 and 43, *sp.*). The separation continues, and immediately after the last ecdysis a communicating space is observable extending around the central core of cells within the germinal center and in between the two layers of the ventral cell strand. The germinal center and ventral cell strand have consequently formed a common duct. This duct is the gonadal portion of the vas deferens (figs. 44, 48). The follicle ducts join this duct at the upper dorsad portion.

It is now that we see the significance of the ingrowth of fibers visible at the beginning of the second instar, separating the germinal center from the ventral cell strand in limited areas. As the space between the lining epithelium and the central core of cells of the germinal center increases this central core or mass of cells is seen to be associated with the ingrown fibers. The latter penetrate into the mass and in some instances pass through it, reaching the opposite wall (figs. 42 and 48, *ingr.*). In this manner, the central core of cells is definitely suspended in the duct which is formed.

Cephalad the duct ends blindly, a short distance beyond the region where it is joined by the most anterior follicle ducts. This cephalic portion does not contain any central core at its blind extremity (fig. 46). In fact, this hollow blind portion develops from cells, already noted, which continue cephalad to the embryonic genital rudiment and represent the continuation of the genital rudiment devoid of germ cells. Caudad, the duct continues as the vas deferens proper, a hollow tube without a central core of cells.

This duct into which the follicle ducts empty has been called the 'vas deferens' by Sutton ('00) and Davis ('08) and the 'seminal duct' by Uvarov ('28). But it follows from the above that it is not strictly homologous with the vas deferens in all respects, due to the presence of the modified germinal center in its development and final structure, nor is it homol-

ogous with the seminal duct when this term is applied to include what is commonly called the vas deferens.

10. The formation of the spermatocysts. The primary spermatogonia remain as such so long as they retain their intimate association with the apical cell. When this relationship has been destroyed, the germ cells become secondary spermatogonia. A description of the manner by which this occurs may be facilitated by a review of the cellular conditions obtaining in the apical end of the follicle during the instar development.

At the end of the first instar the apical complex is in contact with the outer membranous covering of the follicle by means of the surrounding capsular elements of the complex (figs. 37 and 38). During the second instar other cell groups begin to occupy the intervening space between the capsular elements and the covering membrane. These are more often noticeable on one side of the young follicle viewed in longitudinal section, due to the eccentric position which the apical complex assumes as it is forced to the side by the formation of secondary spermatogonia and connective-tissue elements. During the later instar periods, the apical complex generally occupies the extreme distal position in the follicle with its capsular cells partly in contact with the external membrane. From this region of contact,[20] extending proximally, there is an intervening space between the capsular cells and the outer membrane, filled by young spermatocysts and connective-tissue cells. The young spermatocysts arise in one of two ways.

In the division of a primary spermatogonium, the spindle may form so that the long axis is parallel to the free surface of the spermatogonium (i.e., the surface more distant from the apical cell). When this occurs, the resulting daughter cells remain in contact with the apical cell, though one may retain a more intimate contact than the other. Or the long axis of the spindle may form perpendicularly to the free sur-

[20] The apical complex is not rigidly fixed in the growing follicle tip, but shifts its position as a result of the growth of the tip as a whole.

face of the spermatogonium. Of the resulting daughter cells of such a division, one retains the position of the original mother spermatogonium in contact with the apical cell, and the other is situated toward the outside, on the periphery.

As a result of the former method of division, certain primary spermatogonia come to assume a position more distant from the apical cell than the general mass of primary spermatogonia. They appear as though they were being crowded out of the circle of intimate contact with the apical cell. The capsular cells retain their connection with the apical cell during this process. Consequently, as this crowding out continues, in the case of a single primary spermatogonium, the capsular cells and processes actually come to surround it. When this occurs, it is spoken of as a secondary spermatogonium, and is the mother cell of a spermatocyst.

In the second method of division, one of the daughter cells directly passes off into the surrounding environment, and the capsular cells, as above, surround it. It is a secondary spermatogonium. There is no essential difference between these two methods of secondary spermatogonium and spermatocyst formation other than that the latter is more direct than the former.

The divisions occurring in the cells of the younger cysts are simultaneous. In the older cysts, however, it is not uncommon to observe cells on different sides of the cyst compartment in different stages of mitotic activity.

It is evident from the foregoing that the younger cysts are situated in the distal end of the follicle. At the time of the third instar the follicle is a well-developed structure with the older cysts located near the follicle ducts, whereas successively younger cysts are distributed distally. The question arises: How is this distribution effected?

Sutton ('00) refers to this distribution as "the riper cysts being forced toward the vas deferens by the growth of those beyond," and Davis ('08) writes similarly, as follows: "This is, of course, due to the fact that existing cysts are continually being forced toward the vas deferens by the formation

and growth of new cysts at the distal end." While this view may be tenable to one who observes the mature follicle, it is not so plausible when the development of the follicle is under observation.

As described above, the internal network of connective-tissue cells and their processes constitute a framework. This framework is set up in the very young follicle. It is doubtful that this framework is altered other than by a growth process or that it permits a movement of cysts during the instars other than by such a process.

Further, the apical end of the follicle grows as well as other regions of the follicle. Whenever the covering fat-body and the dorsal diaphragm block its growth dorsad, it turns laterad, continuing its growth in that direction. This is shown by the positions which the follicles assume during the later instars. It is highly probable that during this process the cysts already formed are left behind by the growing apical end. As a result the older cysts occupy positions in the follicle nearer the follicle ducts. That is, the cysts formed at the beginning of the second instar are located near the follicle ducts, while those formed later are distributed farther away toward the distal or apical end.

11. Spermatogenesis in relation to the instars. The secondary spermatogonia formed at the beginning of the second instar undergo mitoses throughout the second, third, and part of the fourth instars. These are the gonial divisions. In the fourth instar the gonia of these cysts enter upon the growth period and are then spoken of as first spermatocytes. The various stages peculiar to the growth period are experienced during the latter part of the fourth and through the fifth instars. At the beginning of the sixth instar the maturation divisions occur with the resulting production of spermatids. Shortly after the adult molt, these spermatids complete their metamorphoses into active sperm elements.

In the meantime, the spermatogonia of successively younger cysts are passing through some phase of spermatogenesis, depending upon the time of formation of the mother cell of

each cyst. At the end of the sixth instar, therefore, each follicle presents a résumé of the spermatogenesis phenomena from the formation of secondary spermatogonia at the apical end to the differentiating spermatid in the region of the follicle duct.

For the details of spermatogenesis in the Acrididae, see Davis ('08), McClung ('00, '27), and Wenrich ('16 and '17).

12. The growth of the follicles. A measurement of the follicles during the instars from the second to the adult condition indicates that the rate of growth of the follicles is greatest during the second and fourth instars. The third and fifth instars present periods of slower growth, and during the sixth instar the growth is still slower.

13. The differentiation of the follicle ducts. Each follicle duct is formed by a growth of cells from the germinal center, in column-like formation, against the apical complex, and by an ingrowth of the outer limiting membrane over this column. This results in a narrow tube closely packed with epithelial cells. Some of these cells migrate into the developing follicle, where they are ultimately modified into supporting elements.

The remaining cells later become arranged upon the connective-tissue wall of the developing duct, forming a layer of low columnar epithelium. A distinct lumen does not appear until just previous to the adult molt (figs. 49 and 50).

14. The adult testes. A count of the follicles in the adult testes shows an approximate total of 188. They are held together by a covering of fatty tissue which surrounds the testes. This fatty covering extends between the follicles, filling the interfollicular spaces. Where the follicles are contiguous, the intervening fatty tissue is in the form of a much flattened membrane.

The follicles are joined to each gonadal vas deferens over a longitudinal extent averaging 2.8 mm., and from the region of attachment extend dorsad through slightly curved arcs (fig. 47). The follicle ducts may join the vas deferens singly or in groups of two or three.

The fatty covering causes the testes at the time of the adult molt to appear orange in color, but the follicles themselves are translucent.

The testes average 5.86 mm. in length, 3.92 mm. in width, and reach a height of 1.95 mm. They are situated in segments 1 to 4, inclusive.

SUMMARY AND DISCUSSION

A. Summary

1. The life cycle of Melanoplus differentialis in this locality extends through a period of one year. Development begins immediately after oviposition and continues through the fall and winter months. The revolution of the embryo occurs in the spring. There follows a period of rapid differentiation of embryonic rudiments. Hatching occurs in the late spring, and the postembryonic development extends from the time of hatching to the late summer. Copulation and oviposition occur in the late summer and the early fall.

2. Sex is visibly differentiated during the revolution period.

3. The development of the testes:

a. The genital rudiments at the beginning of the postrevolution period in the case of the male consist of two somewhat rounded structures extending from the third to the seventh abdominal segments.

b. The differentiation of this rudiment occurs in the following manner:

The isolation of small groups of germ cells (cell nests) results from the partitioning activity of differentiating connective-tissue cells. Then one of the surrounding indifferent cells of such a cell nest moves inward between the germ cells, sending processes among the germ cells. It ultimately comes to lie in the center of the germ cell group. The processes of this central cell—the apical cell—unite with the processes of the surrounding capsular cells. The designation apical complex is given to this group of apical, germ, and capsular cells.

Epithelial cells from the germinal center grow against each of these apical complexes coming in actual contact with the

capsular cells of the complex. While this is being accomplished, the outer limiting membrane of the genital rudiment grows in between these cell groups, ultimately reaching the germinal center. This growth ceases, however, before the integrity of the latter is destroyed. The outer limiting membrane thus forms the connective-tissue covering of the follicle and its duct. The rudiment of the follicle is in this manner established at the beginning of the second instar.

Secondary spermatogonia are formed at this time and continuously throughout the remaining instars. Those formed at the beginning of the second instar experience the gonial divisions from the time of formation to the first part of the fourth instar. They enter the growth period during this instar and undergo the maturation divisions at the beginning of the sixth instar.

It appears that the growth of the follicle is relatively rapid during the second and fourth instars, less rapid during the third and fifth instars, and slower during the sixth instar.

The ventral cell strand, which at the beginning of the postrevolution period is joined to the body of the genital rudiment laterad near its ventral edge, moves mesiad during the course of the postrevolution period and at the beginning of the first instar occupies a position ventral to the rudiment near the mesial edge. This structure and the germinal center ultimately form the gonadal portion of the vas deferens. The epithelial core of the latter is formed by an excess proliferation of cells in the germinal center and possibly from some cells of the ventral cell strand.

4. In the adult testes there are approximately 188 follicles. The ducts of these follicles may join the gonadal vasa deferentia singly or in groups of two or three. The follicles are bound together by means of a covering of fatty tissue which is also inserted between the follicles.

Summary of genital-rudiment differentiation in the male

Embryonic rudiment	*Adult structure*
Terminal filament	Part of connecting filament of testes
Dorsal cell mass	Cells become incorporated in the central cell mass as indifferent elements. Persists in the female
Central cell mass	
a) Primordial germ cells	*a*) Primordial germ cells become primary spermatogonia
b) Indifferent cells	*b*) Some aid in forming connective-tissue framework of the follicle—some differentiated into apical cells
Germinal center	*a*) Some cells contribute to connective-tissue framework of the follicle
	b) Some cells form epithelial lining of follicle ducts
	c) Some cells form epithelial core of gonadal vas deferens
	d) In part contributes to gonadal vas deferens
Ventral cell strand	*a*) Cells may act as reserve supply of indifferent elements
	b) As a whole contributes to formation of gonadal vas deferens
Outer limiting epithelial membrane	Becomes covering connective-tissue membrane of the follicle, follicle duct, and gonadal vas deferens
Fat-body rudiment	General fatty covering of the testis, interfollicular packing tissue. (This consideration of the fat-body is given only as related to the testis)

B. Discussion

1. Origin of the term 'apical cell.' The phrase 'die Apicalzelle der Insekten-keimdrüsen' used by Grünberg ('03), because of the position of this cell in the apical or blind end of the follicle, marked the beginning of the designation 'apical cell' in zoölogical literature. Previously, it had been called 'Verson's cell,' e.g., by Toyama ('94), after its supposed discoverer. But Cholodkovsky ('05) pointed out, "Nicht Verson, sondern Spichardt hat entsprechende Gebilde zuerst gefunden und unter dem wenig passenden Namen 'Keimstelle' beschrieben" (Spichardt, '86).

2. The origin of the apical cell. This has been a subject of considerable interest and discussion. Toyama, working on

Bombyx mori, came to the conclusion that the apical cell is derived from one of the follicular cells. Grünberg, working on the same form, described it as "eine umgewandelte Geschlechtszelle," and stated that "Wir müssen die Apicalzelle auffassen als eine ursprüngliche Keimzelle, die jedoch schon früh Charakter als solche verliert, um eine andere Funktion zu übernehmen." La Valette St. George ('97) reached a similar conclusion, and Munson ('06), in a study of Papilio rutulus, regarded it as a germ cell.

Grünberg, in his description of the origin of this cell in the silkworm, writes:

> Ungefähr in der Mitte dieser Plasmaanhäufung, der Hüllmembran des Hodenfaches genähert, liegt ein Kern, der sich in keiner Beziehung von den Kernen der Spermatogonien unterscheidet. Das ganze Gebilde, welches durch seine Grösse auffällt, macht den Eindruck einer selbständigen Zelle. Dieselbe zeigt im Allgemeinen eine flach kegelförmige Gestalt. Sie beginnt mit stark verbreiterter Basis an der Innenfläche der Hüllmembran und ragt mit ihrer Spitze in das Innere des Hodenschlauches vor, wo sie vermittels eines mehr oder weniger breiten Plasmastranges kontinuirlich in das den Raum des Hodens erfüllende Plasma übergeht. Dagegen ist das Plasma der Zelle selbst in allen Fällen durch seine grössers Dichte und seine dadurch bedingte dunklere Färbung von dem Plasma der Spermatogonien zu unterscheiden.

Toyama ('94 a) writes regarding the origin of the apical cell:

> Into each of these depressions, a follicular cell enters which soon enlarges and loses its cell wall. The genital elements already present now arrange themselves around this follicular cell and in later stages it is clearly to be seen that there is a protoplasmic communication between this invaginated follicular cell and the genital cell. I have not found, as Verson states, this central cell in the state of division in tracing it from the time when it is recognizable until the copulation of the imago.
>
> From all these, we come to the conclusion that the single large cell found in the blind end of the follicle is not a germ cell, as Verson states, but it corresponds rather to the supporting cells of the testes of Vertebrates, or the rhachis of the Ascaris egg string in its function.

Viewing these divergent conclusions and the process of apical cell differentiation in M. differentialis, we are able to make the following comparisons: First, the initial stages of the differentiating process in Bombyx mori and M. differentialis are somewhat similar. In both forms a cell situated near or in contact with the outer covering membrane of the forming follicle rudiment pushes in among the germ cells. The later stages of this particular part of the process are not alike, however, for in B. mori an invagination occurs ('Einsenkung' Grünberg) of the follicle rudiment membrane behind the apical cell, forming a connection with this cell, while in M. differentialis this does not occur. In the latter the apical cell soon loses contact with the outer membrane of the rudiment. Secondly, regarding the character of the differentiating apical cells we note that the cytoplasm in both forms is distinct from the cytoplasm of the germ cells. But the nucleus of the differentiating apical cell in B. mori is similar to the nuclei of the germ cells, whereas in M. differentialis the nucleus of the differentiating cell is identical in all respects with the nuclei of the surrounding connective-tissue elements. Further, we are unable to observe any disappearance of the cell wall in M. differentialis as Toyama observed in B. mori. Again, in harmony with the observation of Toyama (and of Munson in the case of Papilio rutulus), in M. differentialis we are unable to observe any divisions of the apical cell from the time of differentiation to the sexually mature adult. Finally, Toyama speaks of the germ cells as arranging themselves around the apical cell. In M. differentialis, to the contrary, they appear to be manipulated by the connective-tissue elements. This is in harmony with the apparent objective of the germ cells throughout the history of the germ gland, which is one of multiplication and ultimate differentiation into sperm elements; while that of the connective-tissue cells, including the apical cell, one of preparing the proper conditions so that this rôle of the germ cells may continue uninterrupted. Doubtless, the presence of the germ cells influences the general course of the differentia-

tion processes of the gonad, but this is probably indirect, possibly through the so-called inductive action so beautifully demonstrated by work on the Amphibia (Brachet, '27).

Accordingly, it appears that the history of the differentiating process of the apical cell in these forms is similar in some, and dissimilar in other, respects. But this may be expected, in view of the generic differences.

3. The function of the apical cell. The function of this cell cannot be stated as definitely as its origin. Verson ('89, '94) believed it to be the progenitor of the germ cells, through a process of amitosis, and Munson ('06) also regarded it as the cell which produced the germ cells and other cells of the follicle. The latter regarded the apical cell as the 'grandmother stem cell' and the progenitor of the 'mother branch cells.' The 'mother branch cells' in turn gave rise to the 'primary spermatogones,' each of the latter producing the cells of a cyst. Munson states that he has "never seen the grandmother stem cell divide," and consequently, in "considering the intimate relation between the grandmother stem cell, the mother branch cells, and the cortical nuclei, a primitive germinal syncytium is suggested, out of which the cortical nuclei are being organized into cells, while the mother branch cells are completely differentiated with the exception of their protoplasmic connection with the stem cell" (pp. 65, 66).

The writer, not having studied the relationships of these cells in Papilio rutulus from which the considerations of Munson are deduced, does not feel justified in commenting other than to suggest that the figures and descriptions of Munson regarding the relationship of the 'grandmother stem cell' and 'mother branch cells' resemble the relationship between the apical cell and primary spermatogonia in M. differentialis.

In regard to the function of the apical cell Grünberg (p. 337) writes:

> In Wirklichkeit steht die Apicalzelle jedoch während ihrer ganzen Vegetationsperiode in unmittelbarer Verbindung mit der Hüllmembran, und diese Verbindung dürfte jedenfalls für ihre Ernährungsthätigkeit von grosser Bedeutung sein.

As a result, on page 378, he mentions two possible functions:

> Ihre Thätigkeit als solche kann eine doppelte seine; durch Aufnahme von Material und Verarbeitung desselben übt sie eine assimilirende Thätigkeit aus; ausserdem kann sie durch selbständige Produktion von Nährsubstanz die Bedeutung einer secernirenden Nährzelle gewinnen.

The function of the apical cell as a progenitor of germ cells is definitely out of harmony with the facts so far as M. differentialis is concerned. Moreover, its rôle as an assimilating cell and nurse cell is doubtful. At the time of the differentiation of this cell in M. differentialis it has an intimate relation with the outer limiting membrane of the genital rudiment. This is similar to the relationship which Grünberg, Verson, and Toyama describe. However, this is only a transitional relationship in M. differentialis, and when the follicle rudiment is definitely established, the apical cell possesses this relationship with the outer limiting membrane of the follicle only in an indirect manner through the capsular cells and their processes.

The necessity for this assimilative and nurse-cell rôle in M. differentialis is questionable. The germ cells undergo mitotic activity in the genital rudiment previous to any relationship with the apical cell as such. And this mitotic activity proceeds much the same after the relationship with the central cell or apical cell is established. Why is it necessary to assume that a condition arises when the germ cells need a special cell to control or govern their metabolism, either directly or indirectly? On the other hand, its relationship with the surrounding capsular cells and the primary spermatogonia suggests that it forms an integral part of a supporting mechanism one of the main functions of which is the production of secondary spermatogonia in as large numbers as possible.

In consideration of the idea of the apical cell as a supporting cell, it is to be noted that there is no apparent distinction between primary spermatogonia and newly formed secondary

spermatogonia. The difference between the two is one of structural relationship. When a primary spermatogonium loses its contact with the apical cell, it becomes surrounded by connective-tissue elements and is then regarded as a secondary spermatogonium and the mother cell of the future gonia in the spermatocyst. Consequently, if the apical cell were removed from the apical complex, there is the possibility that the number of secondary spermatogonia and subsequent spermatocysts formed would be the same as the number of primary spermatogonia surrounding the apical cell at the time of its removal. But as long as the apical complex remains intact, the production of secondary spermatogonia and spermatocysts would theoretically continue indefinitely. The mechanical exigencies of a particular situation apparently demand the presence of the apical cell as part of a supporting system. The presence of the apical cell in the center of the group of primary spermatogonia probably prevents the latter from becoming secondary spermatogonia, and the subsequent breakdown of a machine which is producing secondary spermatogonia, and ultimately, the sperm elements.

The apical cell was regarded as a supporting cell by Toyama ('94) and seemingly so by Erlanger ('96). La Vallette St. George ('97) believed it to be a 'Stütz- und Ernährungszelle,' while Mohr ('14) wrote: "Wir haben sie niemals sich teilen gesehen, und ihr ganzer Charakter gibt den Eindruck einer etwas speciell differenzierten Bindegewebszelle"; and Schellenberg ('13) concluded: "Zu den somatischen Zellen muss auch die Versonsche Zelle gerechnet werden" and "Ob sie nur als Stütz- oder auch als Nährzelle der jungen Spermatogonien dient, mag dahingestellt bleiben."

We have, therefore, a range of opinion regarding the function of the apical cell—as a progenitor of germ cells, an assimilating and nurse cell, a supporting and nurse cell, and a supporting cell.

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EXPLANATION OF PLATES

A camera lucida was used as an aid to all drawings excepting figures 7 to 14, inclusive. All figures were reduced one-half in reproduction. Figures 21, 23 to 41, 49, and 50 were drawn at a magnification of 1650. The initial magnification of the remaining figures drawn from sectioned material is given in the explanation accompanying the figures.

ABBREVIATIONS

am., amnion
ap., apical cell
ca.c., capsular cell of apical complex
ca.cy., connective-tissue cell of cyst wall
c.c., central core of follicle
c.c.m., central cell mass
ce., cercus
coe., coelom
c.f., connecting filament of testes
c.n., cell nest
d.c.m., dorsal cell mass
d.d., dorsal diaphragm rudiment
e.c., epithelial core of gonadal vas deferens
el., elastic fiber bundle
e.st., eighth abdominal sternite
f., fat-body
f.d., follicle duct
fo., follicle
f.rud., follicle rudiment
g.ce., germinal center
gp., genital plate
g.r., genital rudiment
g.v.d., gonadal vas deferens
h., heart
hyp., hypodermis
ind.c., indifferent cell
ingr., ingrowth of outer limiting membrane
l.m., lateral margin
lu., lumen
o.l.m., outer limiting membrane
p.g.c., primordial germ cell
p.sp., primary spermatogonium
se., serosa
spn., splanchnopleure
s.sp., secondary spermatogonium
s.st., seventh abdominal sternite
t.f., terminal filament.
um., umbilicus
v.c.s., ventral cell strand.
v.d., vas deferens
y., yolk
y.cyst., young cyst
VII, *VIII*, *IX*, *X*, seventh, eighth, ninth, and tenth abdominal appendages

PLATE 1

EXPLANATION OF FIGURES

1 Female; caudal end of abdomen, showing abdominal appendages at beginning of revolution phenomena.

2 Male; same.

3 Female; caudal end of abdomen showing abdominal appendages during latter part of yolk circumcrescence.

4 Male; same.

5 Female; as above, at beginning of postrevolution period.

6 Male; same.

7 Female; as above, shortly after pronymphal or intermediate molt.

8 Male; same.

9 Female; as above, at beginning of third instar.

10 Male; same.

11 Female; adult, showing fully developed ovipositors.

12 Male; adult, showing fully developed genital plate.

13 Female; same as 11, lateral view.

14 Male; same as 12, lateral view.

15 Camera-lucida drawing of ventral view of embryo just after completion of the revolution arcs.

16 Same as 15, lateral view, showing relation of embryo to amnion and serosa.

17 Dorsal view of embryo just after engulfment of the yolk.

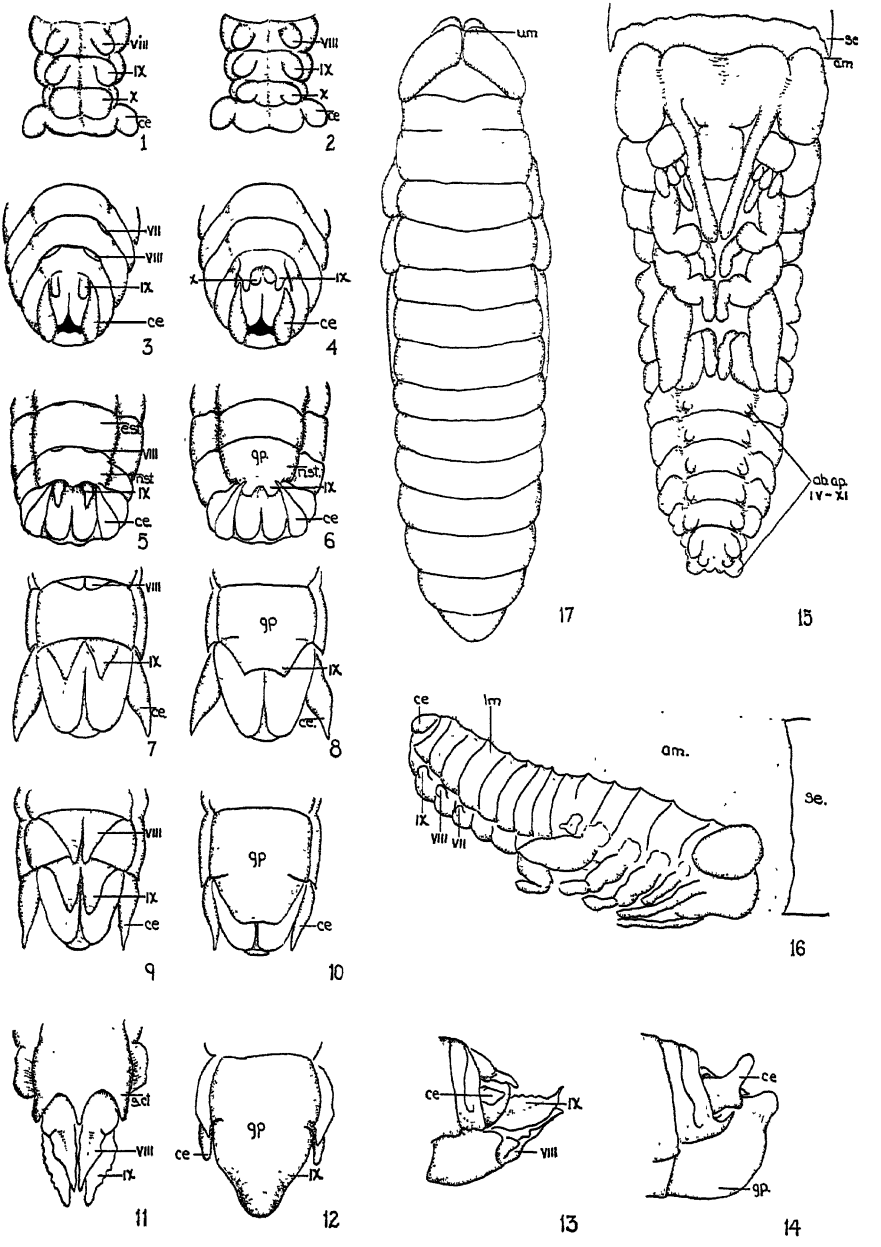
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PLATE 2

EXPLANATION OF FIGURES

18 Outline drawing of transverse section through seventh abdominal segment of embryo immediately after the completion of the revolution ares. × 170.

19 Transverse section through right genital rudiment and related structures as shown in figure 18. × 780.

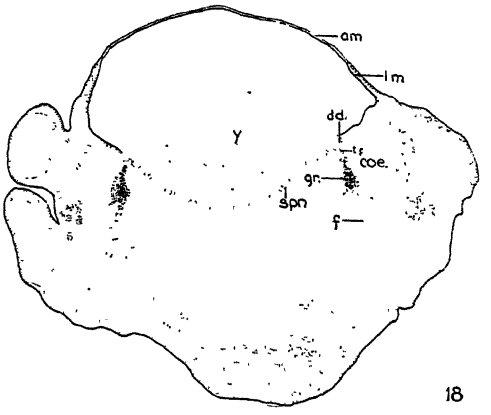
20 Transverse section through genital rudiment and related structures upon reaching the dorsal region of the embryo. × 380.

21 Splanchnopleure, dorsal diaphragm rudiments, and connecting filament of the testes at the time of their final separation at the end of the revolution phenomena.

22 Genital rudiments and related structures at the beginning of the postrevolution period. × 780.

49 Transverse section of developing follicle duct near close of sixth instar.

50 Same, shortly after adult molt.



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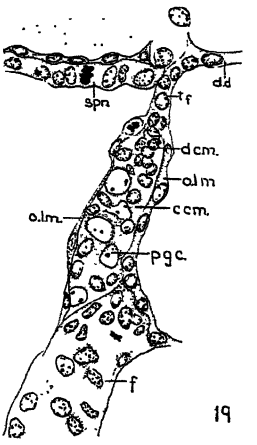

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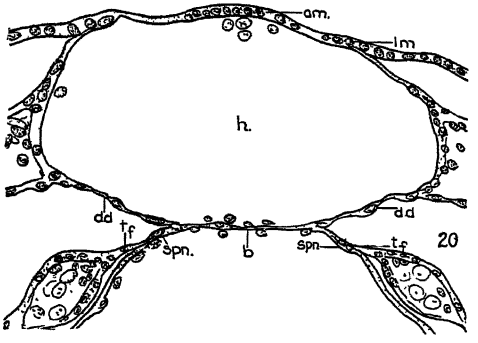

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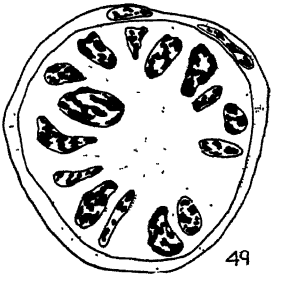

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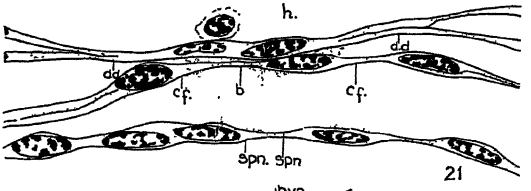

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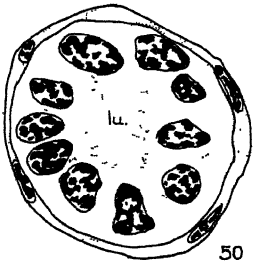

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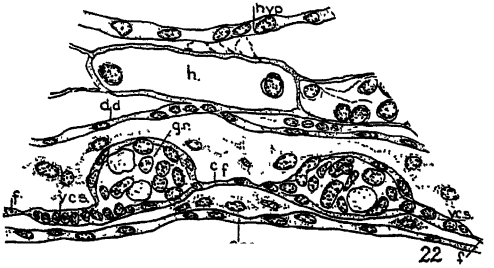

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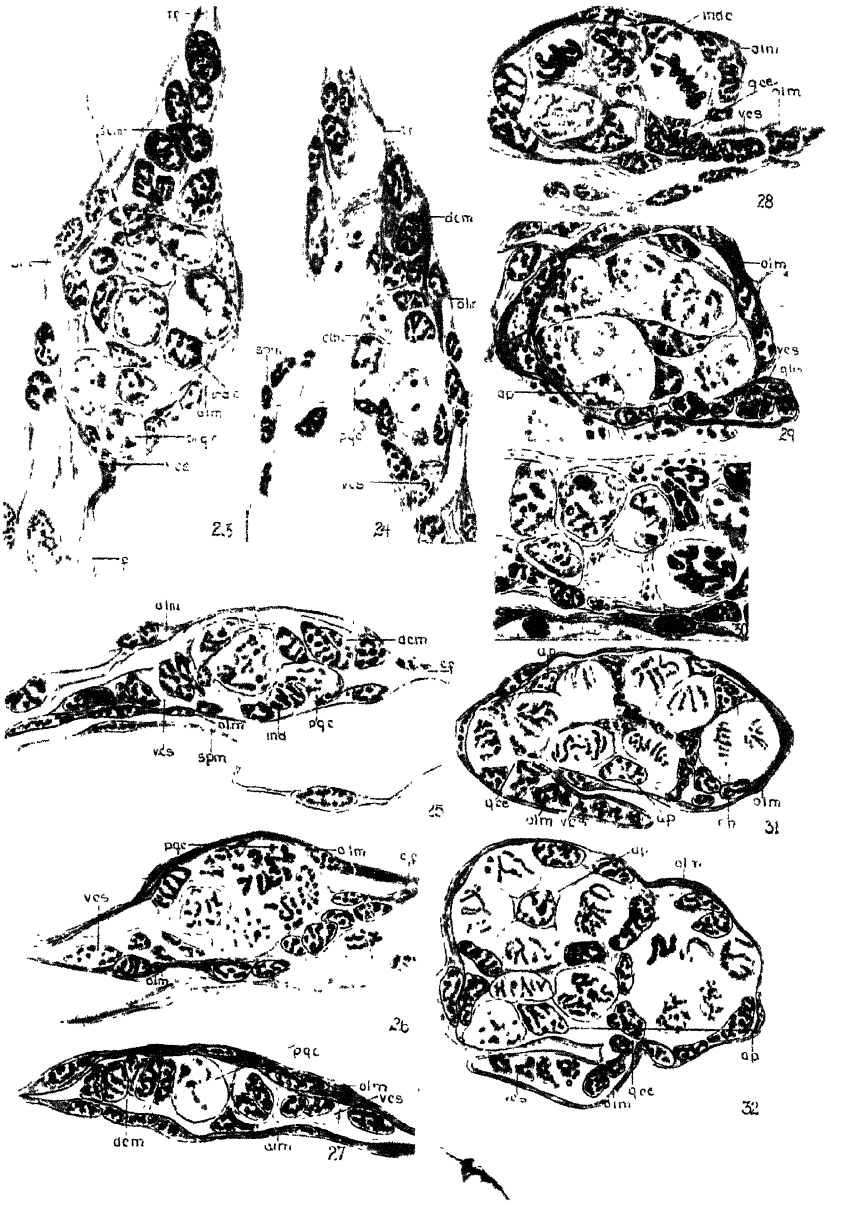
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PLATE 3

EXPLANATION OF FIGURES

23 Transverse section through genital rudiment in sixth abdominal segment immediately after completion of the revolution arcs.

24 Transverse section through genital rudiment during its migration dorsad.

25 Transverse section through genital rudiment as it approaches final dorsal position. The dorsal cell mass is disappearing as a definite structure, characteristic of the male at this time.

26 Section through male genital rudiment at beginning of the postrevolution period.

27 Section through female genital rudiment at beginning of the postrevolution period. Note that the dorsal cell mass remains.

28 Section through male genital rudiment at middle of the postrevolution period.

29 Section through male genital rudiment near close of the postrevolution period.

30 Sagittal section through male genital rudiment at approximately the same stage as in figure 29.

31 Transverse section through male genital rudiment at the time of hatching.

32 Transverse section through male genital rudiment twelve hours after hatching.

PLATE 4

EXPLANATION OF FIGURES

33 Transverse section through cell nest, showing ingrowing apical cell.

34 and 35 Illustrating the same phenomena as in 33.

36 Transverse section through genital rudiment near close of the first instar.

37 Section through follicle rudiment at the beginning of the second instar.

38 Section through developing follicle during first part of the second instar.

39 Section through developing follicle during the latter part of the second instar.

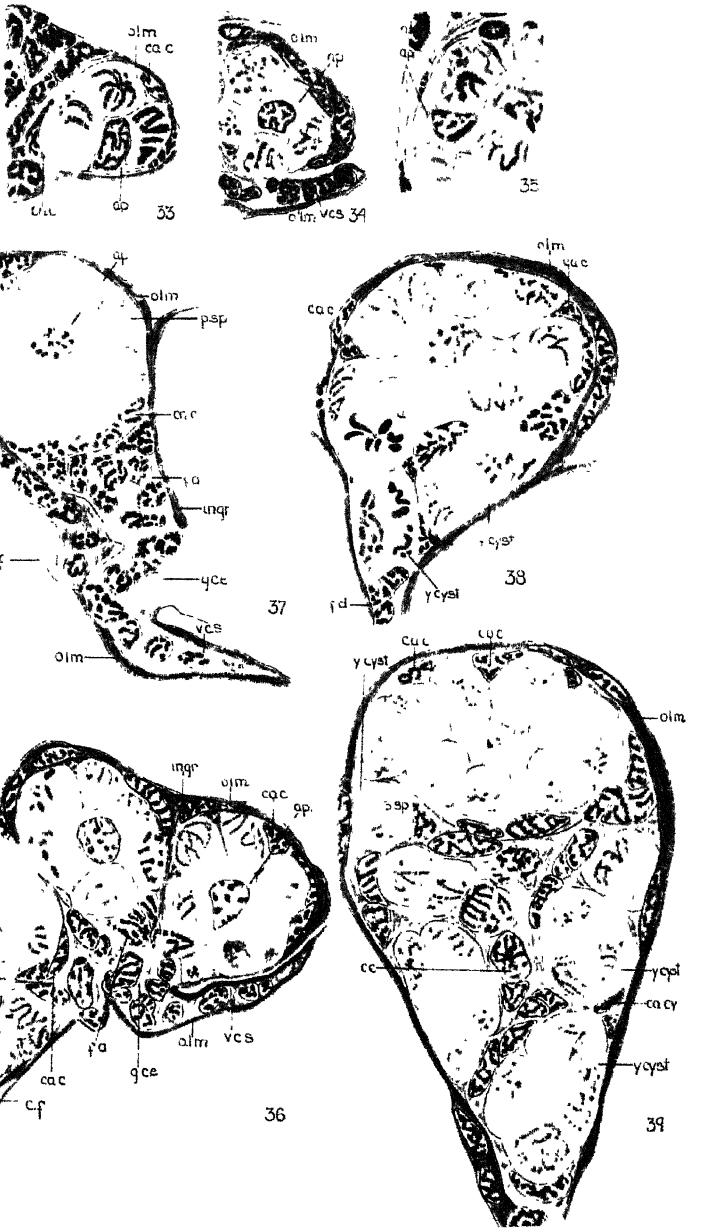
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PLATE 5

EXPLANATION OF FIGURES

40 Transverse section through developing gonadal vas deferens at beginning of fourth instar.

41 Same as 40, showing separation of germinal center and ventral cell strand by ingrowth of outer limiting membrane.

42 Transverse section through gonadal vas deferens at time of last ecdysis, showing ingrowth of outer covering membrane and its connection with epithelial core. × 730.

43 Same as 42, showing epithelial core and forming lumen.

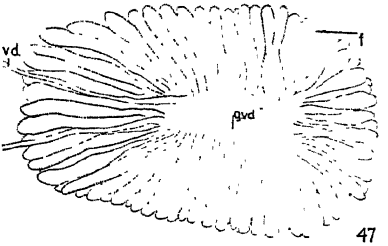
44 Transverse section through gonadal vas deferens seven days after last molt. × 730.

45 Slightly oblique transverse section of a single follicle, showing relation of the central core of the follicle to the surrounding cysts. × 730.

46 Transverse section of gonadal vasa defferentia in their cephalic region. The section to the left cuts through the blind end of the vas deferens. Note the absence of the epithelial core in the latter section. × 170.

47 Ventral sketch of adult testes.

48 Transverse section through gonadal vas deferens, showing epithelial core suspended by ingrowth of the outer covering membrane. × 170.



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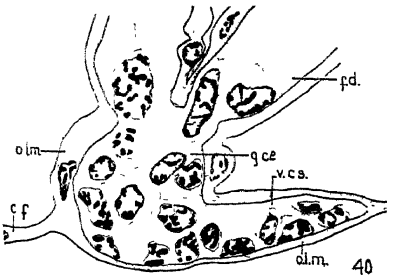

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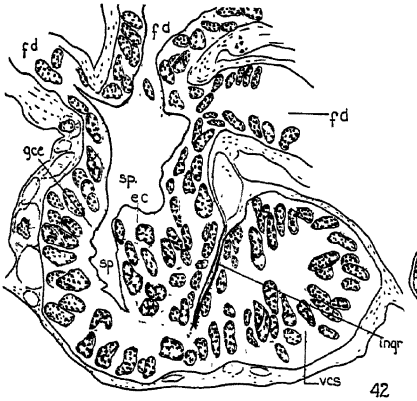

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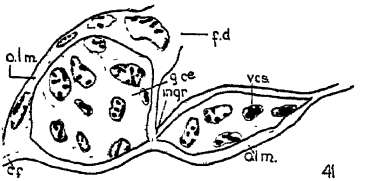

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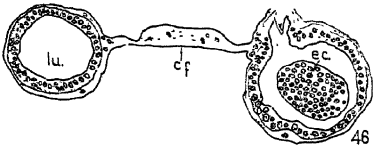

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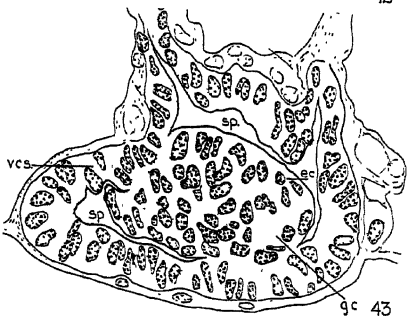

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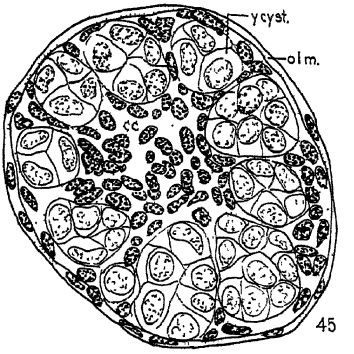

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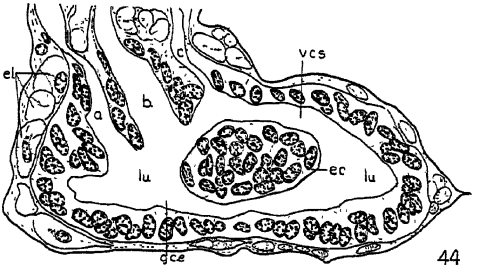

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THE VACUOME OF THE FLAGELLATE CHLAMYDOMONAS

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TWO TEXT FIGURES AND ONE PLATE (EIGHT FIGURES)

AUTHORS' ABSTRACT

A vacuome ('Golgi apparatus') consisting of small globular inclusions has been demonstrated in Chlamydomonas sp. These inclusions may be seen in the living, unstained organism; they are stainable vitally with neutral red; they have been stained vitally with neutral red and then blackened with osmic vapor under direct observation, and they have been impregnated by osmic and silver methods without previous treatment with neutral red.

The reaction of these inclusions to the iodin test for starch suggests that they may play some rôle, possibly one of storage, in the cycle of starch metabolism.

The observations of several workers on free-living flagellates (Phytomastigoda) have shown that certain species, belonging to the orders Chrysomonadida, Cryptomonadida, Dinoflagellida, and Euglenida, possess globular osmiophilic (or argentophilic) neutral-red-stainable inclusions which are similar to the vacuome (Guilliermond, '30; Parat, '28) of metaphytan and metazoan cells and to the so-called 'Golgi apparatus' of Sporozoa (Joyet-Lavergne, '25). In the present investigation similar inclusions have been demonstrated in Chlamydomonas sp. (order Phytomonadida).

MATERIAL AND METHODS

In vital staining, slides were filmed with dilute solutions of the dye in absolute alcohol. Neutral red and Janus green were made up in 1 per cent stock solutions, which were diluted variously in attempts to find satisfactory concentrations. A 1:20 and a 1:15 dilution of neutral red were used in most cases; both are satisfactory, although the initial reaction takes place more slowly in the former. Dilutions of 1:40 or higher were less satisfactory, on account of the longer time

required for staining the vacuome. Both 1.0 and 0.5 per cent solutions of Janus green were unsatisfactory, since there was a tendency for the vacuome to take up some of the Janus green after a few minutes in such concentrations. In 1:10 dilutions the mitochondria were stained in five to ten minutes, while the vacuome usually remained unstained for thirty to forty minutes. Hence, 1:10 and higher dilutions of Janus green are more satisfactory for Chlamydomonas. Mixtures of Janus green (1:2 to 1:10) and neutral red (1:10 to 1:20) in varying proportions were used to stain neutral-red globules and mitochondria at the same time. In these mixtures also it was found necessary to avoid too high a concentration of Janus green.

Using the centrifuge method of concentrating and handling material, permanent preparations were made by the Mann-Kopsch (Weigl) and Kolatchev methods of osmic impregnation and the Da Fano silver method. More detailed accounts of the technique used will be found in other papers (Bowen, '28 a, b, c; Hall, '29 b).

NEUTRAL-RED-STAINABLE INCLUSIONS

In unstained specimens of Chlamydomonas sp. small refractile globules, variable in size and number, are regularly visible in the region of cytoplasm enclosed by the chromatophore. The larger flagellates usually contain more globules than the smaller ones, and the larger the globules the fewer there are in any one specimen. The variations in number and size of the globules are similar to those observed in vitally stained material (fig. A, 1 to 6). The appearance of large globules cannot be due to conditions encountered in sealed-slide preparations, since such variations are seen in flagellates fresh from stock cultures. These variations suggest the possible occurrence of fusion or growth of smaller globules and breaking up of larger ones.

Specimens in the process of being stained vitally with neutral red show such globules which gradually take up the dye (fig. A, 1 to 6). In 1:40 preparations the reaction to

neutral red is slow (twenty to forty minutes), and it is apparent that the normally preexisting inclusions are gradually stained with the dye. In 1:20 preparations similar cytoplasmic globules were stained vitally with neutral red. The globules also varied in size and number in different specimens. At this concentration penetration of the neutral red occurred somewhat more rapidly than in 1:40 preparations, but it was still possible to observe gradual staining of the preexisting globules of the cytoplasm. Fusion of the small globules to form a few larger globules was observed (fig. B, 1 and 2) in continuous observation of a single specimen for fifteen minutes. The preparation was about one hour old when this process began. It should be pointed out that in Chlamydomonas sp. this fusion resulted in the formation of larger globules, and not a network, such as that described by Cowdry and Scott ('28) in Plasmodium.

It might be expected that, in addition to fusion of smaller globules to form larger ones, large globules might break up into smaller ones. Although the latter process was not observed, the common occurrence of globules of various sizes in both unstained and vitally stained material would perhaps favor this view. Although it is possible that such morphological changes in the neutral-red globules might be accelerated by exposure to neutral red, the normal occurrence of such variations in unstained flagellates indicates that exposure to vital dyes is not the only factor involved.

The variations in size, as well as the fusion, of neutral-red globules in Chlamydomonas are similar to those described by Guilliermond ('30) in the vacuome of certain plants. Guilliermond finds that such small granules and large globules are stages in the form cycle characteristic of the vacuome in different types of plant cells. Such a cycle sometimes shows a network stage, which breaks up gradually into large and small globules.

OSMICATION OF GLOBULES STAINED WITH NEUTRAL RED

On exposure of vitally stained material to osmic acid (fig. A, 7 and 8) in sealed-slide preparations, the first effect noted was a diffuse pink staining of the inner zone of cytoplasm. After a few minutes, the globules had, in most cases, assumed a slight bluish tinge, and in some specimens their color was almost violet. After an hour or two the neutral red had begun to fade from the globules and also from the cytoplasm in

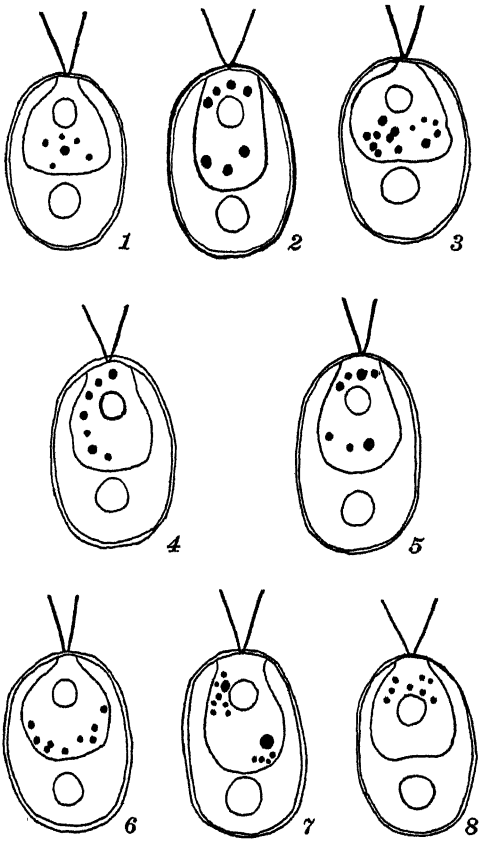

Fig. A 1 to 6. Neutral-red preparations. 7 and 8. Neutral red followed by treatment with osmic acid in sealed-slide preparations. Nucleus, pyrenoid, and chromatophore are indicated in outline; neutral-red globules (1 to 6) and osmiophilic globules (7 and 8) are shown in black. The sketches are semi-diagrammatic.

general. At the end of twelve hours the dye had disappeared from the globules, which appeared as light gray, refractile bodies. After four days, the globules in most specimens were dark gray, and by the end of a week all of them were definitely blackened (summer room temperature).

REACTION TO IODIN-POTASSIUM IODIDE

In preparations stained with iodin-potassium iodide (fig. B, 3 to 5), many specimens showed bluish globules in the inner zone of cytoplasm, and in the majority the pyrenoid also showed a bluish rim varying in thickness in different individuals. The distribution and size of the iodin-stained globules indicate that they are the same as the inclusions normally present and stainable vitally with neutral red. In some specimens the pyrenoid showed a definite blue rim, while the globules, although refractile and clearly visible, were not stained with iodin. In other specimens the globules were blue in color, but the pyrenoid gave no test for starch. And in some specimens in which the globules were stained blue the pyrenoid was apparently absent (fig. B, 5).

These observations suggest that the vacuome of Chlamydomonas plays some rôle in the cycle of starch metabolism, possibly one of storage. If this is the case, it would seem to be in agreement with the view of Guilliermond ('30) that the vacuome, at least in plants, serves as a center of accumulation of various important products of metabolism.

SILVER AND OSMIC IMPREGNATION

Flagellates impregnated by the Da Fano silver method show a variable number of blackened globules (figs. 1 and 2), similar in relative size, number, and distribution to those observed in neutral-red preparations. In some specimens the globules were small and numerous (e.g., twenty); others showed a few, sometimes four or five, larger globules. In two or three specimens a slight impregnation of the nuclear membrane was observed, but such cases were exceptional.

In Mann-Kopsch osmic material, bleached in turpentine, similar blackened globules were observed. Just as in Da Fano material, the globules varied distinctly in size and number; two contrasting examples are shown in figures 3 and 4. The material bleached in turpentine often showed, at the surface of the chromatophore, blackened fibrils which were seldom completely bleached. These structures are possibly to be regarded as fibrillar 'mitochondria.' After bleaching in hydrogen peroxide (figs. 5, 7, and 8) only the globules remained blackened in the majority of specimens. This method of bleaching was not so easily controlled as the turpentine method, and in our material a number of the flagellates showed no blackened inclusions at all, although the globules could be seen as shadowy bodies in the inner zone of cytoplasm.

In our Kolatchev osmic material there is a definite impregnation of the general surface of the cell which persists to a noticeable extent after bleaching in turpentine (fig. 6). The only cells which showed no trace of this surface blackening were bleached completely.

Globular cytoplasmic inclusions were also impregnated by the Kolatchev method; these globules were similar in size range and distribution to those demonstrated by other methods. In addition to the usual inclusions, a few partly blackened granules were seen at the surface of the chromatophore in rare instances; it is possible that these represented incompletely bleached mitochondria.

MITOCHONDRIA

In unstained specimens (fig. B, 6) refractile granules, and sometimes short rods or longer fibrils, were seen, apparently at the surface of the chromatophore. It has been impossible to determine definitely whether these inclusions lie between the periplast and the chromatophore or are just inside the chromatophore. The former interpretation seems more logical, since it is in accord with the known distribution of mitochondria in other chlorophyll-bearing flagellates.

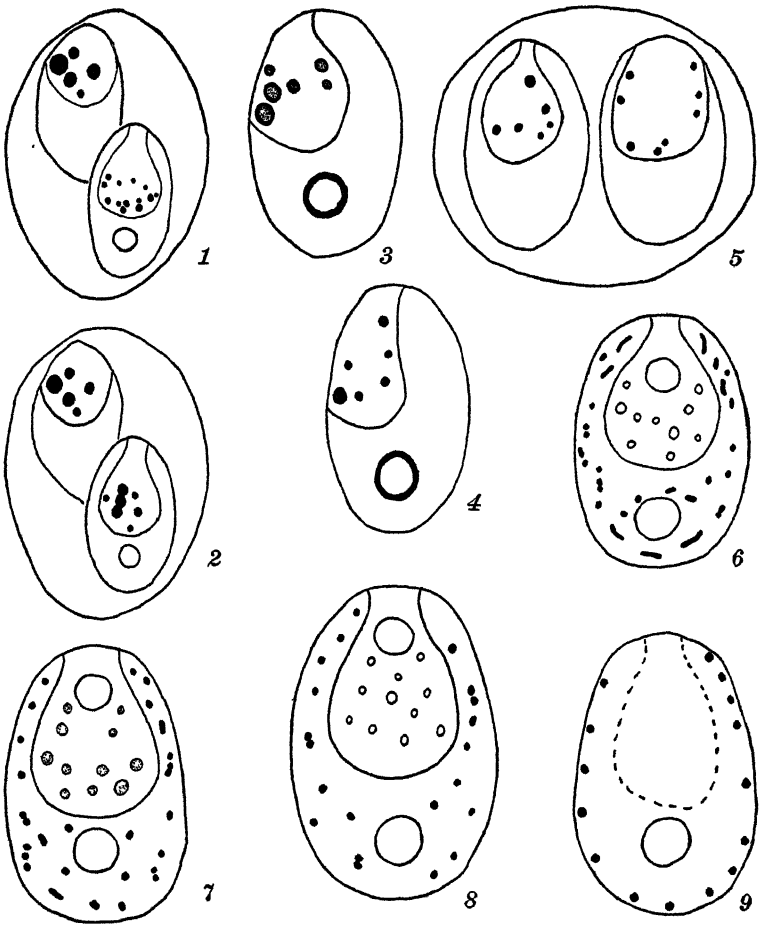

Fig. B 1 and 2. Neutral-red preparations (1:20), two-cell stage; in the lower flagellate (1) there are a number of relatively small neutral-red globules, and fifteen minutes later (2) some of these had fused to form seven larger globules. 3 to 5. Reaction to iodin-potassium iodide. The first two specimens showed a definite reaction of the pyrenoid and the cytoplasmic globules; the third (a two-cell stage) showed no pyrenoids, but the globules were definitely stained blue with iodin. 6. Unstained specimen; refractile globules and rods in region of chromatophore are shown in black; vacuome indicated by circles. 7. Neutral red-Janus green preparation; mitochondria indicated in black; elements of the vacuome stippled. 8 and 9. Janus-green preparations, mitochondria indicated in black; 9 represents an optical section. All figures somewhat diagrammatic.

In preparations stained vitally with a mixture of neutral red (two parts 1:20) and Janus green (one part 1:10) the refractile bodies in the region of the chromatophore become bluish in color within five to ten minutes, whereas the globules of the inner zone of cytoplasm are stained gradually with neutral red (fig. B, 7). If the concentration of Janus green is too high, as was the case in some of the other mixtures used, there is a definite tendency for the vacuome to take up some of the Janus green after the preparation has stood for twenty to thirty minutes or more. The resulting mixed color (a reddish-violet) differs from that of the mitochondria in the chromatophore region; these take up the Janus green alone, and even in neutral-red preparations they have not been stained with neutral red.

When Janus green in 1:10 dilution is used alone (fig. B, 8 and 9), the mitochondria usually react within five to ten minutes, while the vacuome remains colorless at first. After forty minutes or more, however, the vacuome often shows a more or less definite staining with Janus green. With more concentrated solutions of Janus green, the mitochondria are stained rapidly, and the vacuome is also stained after an interval somewhat shorter than for 1:10 preparations.

DISCUSSION

Previous investigations have demonstrated the presence of osmiophilic (or argentophilic) neutral-red-stainable inclusions in the following orders of Mastigophora: Chrysomonadida (Hall, '30 a), Cryptomonadida (Hall, '30 a), Dinoflagellida (Chatton and Grassé, '29), Euglenida (Grassé, '25; Hall, '29 a, '30 a), Protomastigida (Lwoff and Lwoff, '29; Nigrelli, '29). In the present investigation Chlamydomonas sp. (order Phytomonadida) is added to the list. Certain investigators (e.g., Dangeard) have described neutral-red-stainable inclusions in a few other species of the Phytomastigoda. It is to be presumed, although not entirely certain, that such inclusions are identical with the osmiophilic neutral-red-stainable inclusions. It seems obvious, therefore,

that these neutral-red globules are widely distributed in flagellates, and that they show certain similarities in their form and in their reactions to osmic and silver impregnation and to vital dyes.

The resemblance between these inclusions of Protozoa and dispersed stages of metazoan Golgi material has been pointed out by various workers (e.g., Joyet-Lavergne, '25; Hirschler, '27; King, '27; Hall, '29 a, '30 a); likewise, these same inclusions have previously been compared with the vacuome (Grassé, '25; Volkonsky, '29; Hall, '30 b; Nigrelli and Hall, '30). Only in rare instances is there any morphological similarity between the osmiophilic inclusions of Protozoa and the typical Golgi network of a metazoan cell. Cowdry and Scott ('28) observed that in Plasmodium praecox the neutral-red globules fused together to form a simple network, but this seems to be the only instance in which a neutral-red-stainable network has been described in Protozoa. Although the globules of Chlamydomonas may fuse, such fusion results only in the formation of larger globules. A comparison of dispersed Golgi material ('dictyosomes') of metazoan cells with the osmiophilic globules of Protozoa shows, on the other hand, certain obvious resemblances. The two types of inclusions are similar in general form and perhaps in intracellular distribution, and both types are blackened in osmic and silver impregnation.

The osmiophilic neutral-red globules of Protozoa resemble even more closely the elements of the vacuome (Parat, '28; Guilliermond, '30). Elements of the vacuome in some stages may be scattered through the cell, just as are the neutral-red globules of many Protozoa. Both types are similar in form, and they show a definite reaction to neutral red in dilute solutions. Methods of osmic and silver impregnation demonstrate the globular inclusions of Protozoa as well as the dispersed elements of the vacuome. Such similarities seem to justify the use of the term 'vacuome' for these inclusions of Protozoa.

It has been stated by Beams ('30) that, in the acinous cells of the rat pancreas, the vacuome is not impregnated "unless

the tissue be previously stained intravitam by neutral red." The author has concluded also that "the Golgi apparatus and the vacuome are two discrete substances in the acinous cells of the pancreas. . . ." In regard to the obligatory relationship between vital staining and osmication of the vacuome, certain other workers have obtained contradictory results. Dawson ('29), for example, has observed that in skeletal muscle of Necturus the vacuome is demonstrated by osmic and silver impregnation without previous exposure to neutral red. So far as the Protozoa are concerned, there is some justification in either case for classifying the neutral-red globules as Golgi material or as vacuome. The important fact is that, whether they be considered vacuome or Golgi material, they seem to be the only type of inclusions common to all four groups of Protozoa and showing the same essential characteristics. They may be demonstrated by osmic or silver impregnation, independently of any previous treatment with neutral red. In our own experience it has been possible to see similar inclusions in living, unstained Protozoa. Likewise, in dilute neutral-red preparations these normally preexisting globules may often be observed to gradually take up the dye. Furthermore, after being stained vitally with neutral red, the globules have been impregnated with osmic acid under direct observation in sealed-slide preparations. The reaction to osmic is slow under such conditions, and the pictures obtained resemble those observed in material impregnated by osmic and silver methods.

It is possible that neutral red may, under certain conditions, induce the appearance of neutral-red-stainable inclusions in Protozoa. We have tried, however, to avoid this possibility so far as possible by using dilutions of neutral red in which the organisms will live for several days without signs of injury, and our descriptions of these inclusions have been based primarily upon preparations not more than an hour old. In such preparations we have observed no 'induction' of inclusions by neutral red.

While stressing the reaction of these neutral-red globules to osmic and silver impregnation, we must at the same time point out that neither method is specific for the vacuome of Protozoa. It has been found that various other inclusions and organelles may react, occasionally at least, to such methods of impregnation—chromatophores, bacteria and other materials in food vacuoles, surface ridges of the pellicle (ciliates) and periplast (flagellates), contractile vacuoles, large cytoplasmic vacuoles not stainable with neutral red, small granules in cilia and flagella, the stigma of Euglena, the nuclear membrane, mitochondria, pyrenoids, and the general surface of the cell membrane (Chlamydomonas). While it is true that the elements of the vacuome react to impregnation much more consistently than do these other cell structures, it is nevertheless obvious that the reaction to silver or osmic acid can scarcely be regarded as a specific criterion in identification.

A vacuome has been demonstrated in free-living holozoic, saprozoic, and holophytic flagellates and in certain parasitic species. On the basis of the specific distribution of these inclusions, it would be difficult to formulate any general hypothesis as to their particular function or functions in flagellates. In the ciliates Volkonsky ('29) believes that the neutral-red globules enter the food vacuoles and thus should be regarded as zymogen stages of digestive enzymes. Among the flagellates in which a vacuome has been demonstrated, Peranema is holozoic and hence is one to which Volkonsky's concept might be applied. It is not yet certain, however, that there is any definite relation between the vacuome and the food vacuoles in this flagellate.

Even if it be assumed, in the lack of positive evidence, that the vacuome of Peranema does give rise to digestive enzymes, there is the difficulty that these inclusions of Peranema are identical, so far as their morphology, distribution, and reactions may indicate, with the neutral-red globules of the holophytic and saprozoic flagellates. Obviously, it would be impossible to ascribe the same specific zymogenic function

to the vacuome in these other flagellates, and on the basis of present criteria it would be difficult to distinguish any differences in zymogenic or other physiological activities of the vacuome.

Guilliermond ('30) has concluded that the vacuome in plants serves as a center of accumulation of various products of metabolism, especially those soluble in water (proteins, sugars, alkaloids, organic acids, etc.), and that—

> . . . . le vacuome ne doit pas être considéré comme appartenant à la substance vivante de la cellule; il semble résulter d'une forte imbibition suivre de dissolution des substances secrétées par la cellule et qui sont solubles dans l'eau. Il doit être rangé, comme les inclusions de lipoïdes ou d'autres produits, dans ce que l'on désigne sous le nom de paraplasme ou deutoplasme.

On the basis of the present evidence, or lack of evidence—since conditions of metabolism in holophytic and saprophytic flagellates are similar to those in corresponding types of plants—it might be assumed quite readily that the vacuome in flagellates plays some such rôle in cell activities. Such a hypothesis would require of the neutral-red globules only those special properties which would permit the absorption, or adsorption, of various materials occurring in solution in the general cytoplasm. Nor would it seem necessary that the materials so stored should be identical in all flagellates. The reaction of the vacuome of Chlamydomonas to the iodin test for starch seems to support such a hypothesis as that of Guilliermond.

Koehring ('30), in a recent paper on the neutral-red reaction, has concluded that the cytoplasmic granules which stain with neutral red are mitochondria. She states that "The size, movement and orientation of these cytoplasmic staining bodies in various ciliates recall the series of papers by Horning in 1928 on the mitochondria in ciliates and Amoeba. Causey's work in 1926 on Paramecium also definitely establishes these bodies as mitochondria" (p. 70). Koehring has failed to realize that there are two general types of these small cytoplasmic inclusions in Protozoa. It has been shown

by various workers (Chatton and Grassé, '29; Cowdry and Scott, '28; Hall, '29 a, '30 a, b; Hall and Loefer, '30; Joyet-Lavergne, '26; Lwoff and Lwoff, '29; Nigrelli, '29; Nigrelli and Hall, '30; Volkonsky, '29) that, in addition to the mitochondria, there is a second type of inclusions (neutral-red-stainable) designated by some authors as vacuome, by others as Golgi material. These two types of inclusions may be distinguished in vital staining with a mixture of Janus green and neutral red. Furthermore, criteria of size, movement, and orientation are not in themselves reliable enough to identify Horning's mitochondria with the neutral-red globules, and Koehring has presented no additional evidence of her own. In addition, there is no evidence that Horning or Causey, in their work on mitochondria, actually saw the neutral-red globules. It is, therefore, impossible to understand how Causey 'definitely establishes' these inclusions as mitochondria.

It must be pointed out, therefore, that so far as the Protozoa are concerned, Koehring has evidently been dealing, not with mitochondria, but with the vacuome. The correction of this mistake in identity necessarily shifts her emphasis from the mitochondria of Protozoa to the vacuome. In regard to her theory of the function of neutral-red globules, this will require revision of some of her comparisons with the mitochondria of Metazoa.

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PLATE 1

EXPLANATION OF FIGURES

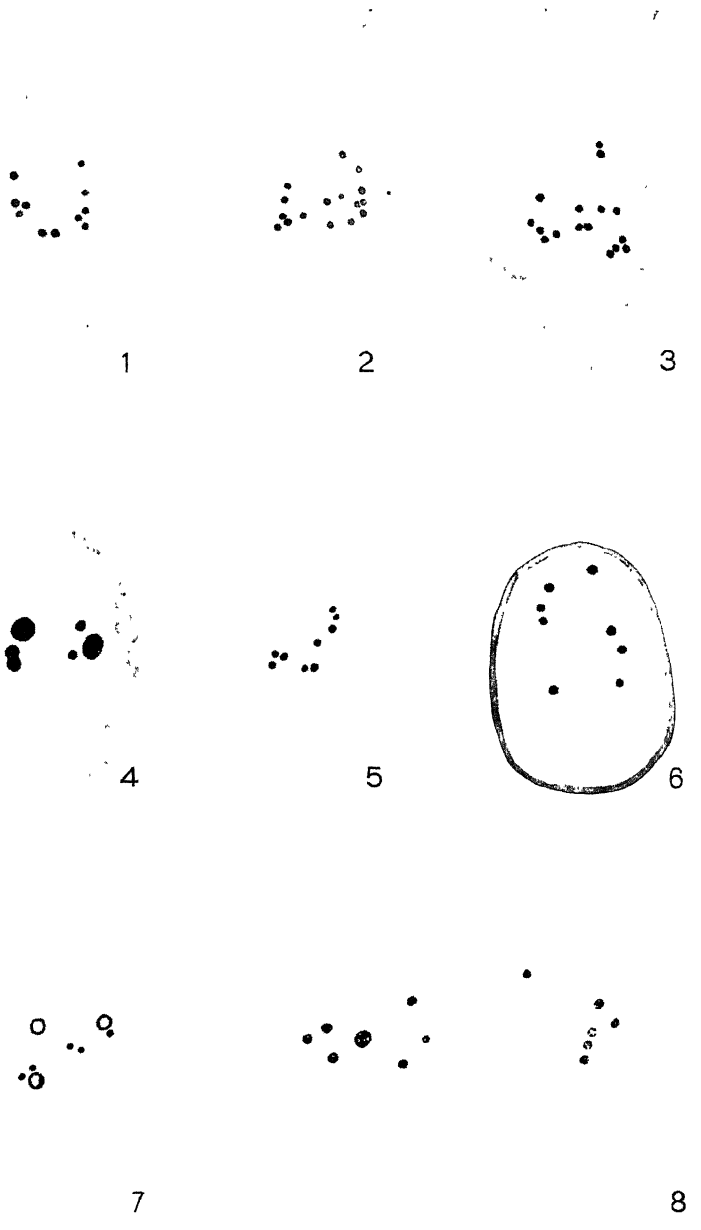
1 and 2 Chlamydomonas sp., Da Fano silver impregnation, showing blackened globules in inner zone of cytoplasm. × 3240.

3 and 4 Mann-Kopsch osmic impregnation, bleached in turpentine. The globules are small and numerous in one specimen, larger and fewer in the other. Note partially bleached inclusions in region of chromatophore. × 3240.

5 Mann-Kopsch osmic impregnation, bleached in hydrogen peroxide; only the globules have remained blackened. × 3240.

6 Kolatchev osmic impregnation, bleached in turpentine. The blackened surface of the cell resists bleaching in this material. × 3240.

7 and 8 Mann-Kopsch osmic impregnation, bleached in hydrogen peroxide. Figure 7 shows one member of a two-cell stage; figure 8, a two-cell stage. In figure 7 the larger globules were bleached so that they appeared to be blackened only on the surface. × 3240.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HISTORY OF THE GERM CELLS IN SPHAERIUM STRIATINUM (LAM.)

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FIFTY-FOUR FIGURES

AUTHOR'S ABSTRACT

Study of living and sectioned material throughout the life cycle shows the germ-cell history from fertilized egg to sexual maturity. This can be divided into the following five periods with definite limits. Original appearance during cleavage, period of inactivity, period of multiplication, maturation, and fertilization. Primordial germ cells of characteristic structure can be recognized just before gastrulation, when there is one large germ cell in the mass of mesoderm on either side of the blastocoel. After one division in each of these two cells, the four daughter cells remain inactive, while the remainder of the mesoderm differentiates, until division is resumed in the developing gonad. An indefinite number of gametes is produced. All are direct descendants of the two original primordial germ cells. Transformation of somatic cells into germ cells does not occur nor do germ cells become somatic cells. Cell lineage shows the two primordial germ cells to be derived from the third division of the paired mesoderm cells which have arisen by an equal division of the fourth micromere produced by cell D of the four-cell stage. Details of meiosis have not been ascertained, because of the small size of the chromosomes, their large number, and the difficulty of fixation. Nothing forecasts which primordial germ cells will become ova and which spermatozoa.

CONTENTS

Introduction 546
Material and methods 546
Fixation 547
Staining 548
Other methods 549
Observations and interpretations 549
Fertilization 550
Cleavage 550
One to four cells 550
First generation of micromeres 555
Second and third generations of micromeres 558
Mesoderm formation and segregation 562
Discussion 567
Period of inactivity of the germ cells 572
Description and location 572
Changes in location 573
Formation of the gonad and its regionation into male and female portions 575
In the early embryo 575
In the late embryo 578
Maturation 580
Discussion 585
Summary and conclusions 589
Literature cited 592

INTRODUCTION

The problem which is the subject of this investigation was brought to my attention in the summer of 1921 during a study of the embryology of Sphaerium striatinum. In this study it became evident that the germ-cell history had not been adequately described in any species of the family Cyrenidae, although the literature contained material regarding certain phases of the subject. It therefore seemed desirable to describe the history of these cells more completely, to relate the scattered observations of previous workers, and to make comparisons with other Mollusca. The work was first concerned with the early history of the germ cells as shown by cell lineage. Later an attempt was made to determine the details of meiosis. It was hoped that differences might be found in the primordial germ cells that would forecast later differentiation into ova or spermatozoa, thus permitting a description of the complete history of the germ cells in a hermaphroditic mollusc.

In this connection, I wish to express my appreciation of the assistance received throughout the work from Prof. W. C. Curtis, at whose suggestion the problem was undertaken. My thanks are also due Prof. M. J. Guthrie for advice regarding cytological methods and to the founders of the Jonas Viles, Jr., Scholarship, which enabled me to work at the Marine Biological Laboratory at Woods Hole, Massachusetts.

MATERIAL AND METHODS

The species studied was Sphaerium striatinum, although comparisons were made with S. ferrisi, with one undetermined species of Sphaerium, and with three species of Musculium. Dr. Bryant Walker, honorary curator of the Department of Molluscs of the Museum of Zoölogy, University of Michigan, verified my identification of material collected near Columbia, Missouri; and Dr. W. J. Clench, curator of molluscs, Museum of Comparative Zoölogy, Harvard University, verified the identification of material collected at Woods Hole, Massachusetts.

All members of the families Cyrenidae and Sphaeridae are hermaphroditic. In Sphaerium striatinum the genital glands have a superficial position in each side of the body extending from the liver to the nephridium. The anterior portion of each gonad is lobed and constitutes the male portion. In large specimens this connects with the single enlarged female region by a short duct. In small specimens the male and female regions are not so distinctly separated. The wall of a single cavity may show maturing spermatocytes at one end and growing oocytes at the other, thus constituting a true ovotestis, while other lobes are definitely either male or female. The female portion of the gland is continued posteriorly as the hermaphroditic duct, which empties into the cloacal chamber near the opening of the excurrent duct from the kidney.

All the stages of development occur in brood pouches, which are closed sacs developed from the lamellae of the inner gills. The formation of these brood pouches and the nutrition of the embryos have been described by Poyarkoff ('01), Schereschewsky ('11), and Groenewegen ('26). In correlation with this development within a brood pouch the trochophore and later larval stages, which are conspicuous in pelecypods having a pelagic mode of development, are reduced in Sphaerium. Reproduction seems to occur throughout the year. Embryos as well as maturing germ cells are found in specimens taken in all seasons, although sperm and ova are not so numerous in winter.

Material was collected chiefly from the vegetation and mud bottoms of ponds in the vicinity of Columbia, Missouri. For comparison I had specimens obtained at Woods Hole and about one hundred slides from Doctor Curtis' collection.

Fixation

In the study of cell lineage whole specimens, gills containing brood pouches, and embryos of various ages dissected from the brood pouches were fixed in either hot or cold Bouin's fluid, Zenker's fluid, Zenker's with formic instead of

acetic acid (compare Guthrie, '26), and corrosive-sublimate-acetic acid. Other fixing fluids have since proved equally satisfactory for the histological features.

In attempting to secure material that would permit counting the chromosomes and observations on the nuclear changes of meiosis, whole animals, embryos, gills, and smears of fresh tissue were fixed in Allen's fluid, Bouin's fluid with formic instead of acetic acid, Carnoy's, Carnoy's with formic instead of glacial acetic acid, the fluid of Carnoy and Lebrun, Flemming's fluid, Zenker's fluid with copper sulphate instead of sodium sulphate, and a fixative used by Doctor Guthrie. This last consists of 15.0 cc. of saturated aqueous solution of picric acid, 1.0 cc. of 3 per cent chromic acid, and 1.0 cc. of formic acid. All these fluids were used at room temperature. Specimens were also teased apart in pond water under a binocular, in tap-water, distilled water, 0.2, 0.6, and 0.9 per cent sodium-chloride solution, and the small pieces thus obtained were transferred to each of the fixing fluids listed above. Such preparations showed no better fixation of chromosomes than whole specimens fixed in the same fluids.

Staining

The material was embedded in paraffin, and sectioned 4, 5, 7, 8, and 10 μ, depending on the features to be studied. Heidenhain's iron haematoxylin and Mayer's haemalum were used with eosin or alkaline orange G in 95 per cent alcohol as counterstain. Such preparations were satisfactory for the general histological features. For observations upon the nucleus the rapid method of using Regaud's haematoxylin gave as good results as the longer method of using Heidenhain's iron haematoxylin. Ehrlich's acid haematoxylin and toluidin blue were also tried. No combination of fixation and staining gave clear chromosome pictures even when sections were bleached with hydrogen peroxide before staining.

Other methods

Embryos in very early gastrula stages and all later stages were dissected from the gills under a binocular microscope and placed in water on a slide for study under higher magnification in both transmitted and reflected light. Camera-lucida drawings of the cleavage and later stages in serial sections were made on transparent paper, and clay models were then constructed, from which figures of the cleavage stages and embryos could be drawn as though from the entire object. For the cleavage stages two drawings of each section were made on the same sheet of transparent paper: one with the upper surface of the section in focus, drawn in red; the other with the lower surface in focus, drawn in black. These drawings were superimposed and models constructed in clay. This method proved satisfactory and required less time than the use of wax plates. In making these models the relative size and position of the cells were carefully followed without attempting to reproduce unimportant details in the actual shape of the cells.

OBSERVATIONS AND INTERPRETATIONS

The primordial germ cells are first recognized as two large cells (fig. 32, *G* and *G.1*), one on either side of the embryo in the early gastrula stage. They lie in contact with a mass of other mesodermal cells on either side of the invaginating endoderm. Meisenheimer ('01 a) describes this production of primordial germ cells as the first differentiation in the mass of cells destined also to give rise to the nephridia, heart, and pericardium. Since the appearance of cells that can be easily recognized as primordial germ cells is thus delayed, their earlier history can be determined only by ascertaining the cell lineage of these two masses of mesoderm cells in which the two primordial germ cells first appear. The work of Whitman ('78 and '87), Wilson ('89 and '92), Conklin ('97), and many others show that cell lineage provides the only means of forecasting the earliest stages of differentiation if distinctive features are not present in cells from which certain organs develop.

Fertilization

The method and time of fertilization of the egg in Sphaerium are not known. I have found no unfertilized eggs in the gills and have found only two fertilized eggs in a stage before the formation of the first cleavage spindle. This confirms the observations of Stauffacher ('93).

Repeated attempts to keep isolated individuals in aquaria to determine whether self-fertilization occurs have been unsuccessful. The animals die within four months. Both ova and sperm are present at the same time in mature individuals. The sperm pass through the ovarian region of the gonad to the outside. Figure 3 shows a spermatozoon at the micropyle of an egg in the hermaphroditic duct, possibly indicating that fertilization occurs there, although such a relationship between sperm and ovum might be accidental, since both pass through this duct to the outside. Ziegler ('85) and Gilmore ('17) state that self-fertilization probably occurs, but offer no evidence other than the anatomical structure of the reproductive organs. Self-fertilization has been demonstrated in some of the hermaphroditic molluscs by Colton ('12 and '22) and Crabb ('27 a and b). This may be the case in Sphaerium, but there is no positive evidence. From existing evidence it is not known whether self-fertilization occurs in Sphaerium.

Cleavage

One to four cells. In designating the cells of the cleavage stages, the following terminology is used: The first four cells are assigned capital letters, *A*, *B*, *C*, and *D*. These are the macromeres. The generations of micromeres are designated by small letters *a.1*, *b.1*, *c.1*, *d.1*, and *a.2*, *b.2.1*, *c.2.2.1*, etc. The first number following the letter is the generation of micromeres from which the cell was derived. In addition to this, the cell receives other numbers indicating the generations of cells that have occurred since the micromere was cut off from the macromere. Thus: $a.1.1 < {}^{a\ 1\ 1\ 1}_{a\ 1\ 1\ 2}$ shows that the cell *a.1.1* is a descendant of the first generation of micromeres arising from *A*, that it is the result of the first division

EXPLANATION OF FIGURES

Figures 4 and 5 and figures 15 to 20 were made from models prepared from serial sections drawn in outline under the microscope. Figures of sections were made with a camera lucida to represent as nearly as possible the appearance of the material on the slide. The magnification after reduction in reproducing the figures is indicated for each figure.

ABBREVIATIONS FOR ALL FIGURES

a.1, *b.1*, *c.1*, etc., first generation of micromeres
a.2, *b.2*, *c.2*, etc., second generation of micromeres
a.3, *b.3*, *c.3*, etc., third generation of micromeres
a.2.1, *b.3.1*, *a.1.1*, etc., descendants of the generation of micromeres indicated by the numeral after the latter (compare p. 550)
A, *B*, *C*, *D*, macromeres of the four-cell stage
aam, anterior adductor muscle
bg, rudiment of the byssus gland
cd, cavity of the hermaphroditic duct
cg, cerebral ganglion
d.4 (*M*), mesoderm-building cell
ect, ectoderm
em, egg membrane
end, endoderm
ent, enteron
es, excurrent siphon
f, foot
G, *G.1*, first two germ cells
g, gonad
gc, germ cells
cgo, cavity of gonad
gi, gill
gr, granules (mitochondria)
h, heart
hpn, anlage of heart, pericardium, and nephridium
hp, anlage of heart and pericardium
is, incurrent siphon
L, liver
lp, lower pericardial cavity
M, mesoderm-building cell
M.1, *M.1.2*, *m.2*, *m.2.2.1*, etc., descendants of *M*
mch, mesenchyme of larval mesoblast
mes, mesoderm
mit, mitochondria
ml, mantle line
n, nephridium
nu, nucleus
nuc, nucleolus
oes, oesophagus
oog, oogonia
ot, otocyst
ovc, cavity of the ovary
p, pericardium
pam, posterior adductor muscle
pb, polar body
per, peritoneum
n per, nucleus of peritoneal cell
pg, pedal ganglion
R, rectum
S, shell
sc, segmentation cavity
sdt, spermatid
sd, sperm duct
sg, shell gland
spc, spermatocyte
spg, spermatogonia
spz, spermatozoa
st, stomach
t, testis
tc, cavity of testis
vg, visceral ganglion
wd, wall of hermaphroditic duct
4c, four chromosomes that precede the others in the anaphase

of this micromere, and that it divided into two cells each of which carries with it the numbers of the parent cell with the addition of another number. The only exceptions to this terminology are the cell *d.4*, which is designated as *M*, since it is a mesoderm-forming cell, and the large cell remaining after the third generation of cells following the bilateral division of *M* which are designated as *G* and *G.1*, since they are germ cells (compare tables 1 and 2). This terminology is similar to that used by Wilson ('92) for Nereis, Lillie ('95) for Unio, and Tannreuther ('15) for Bdellodrilus.

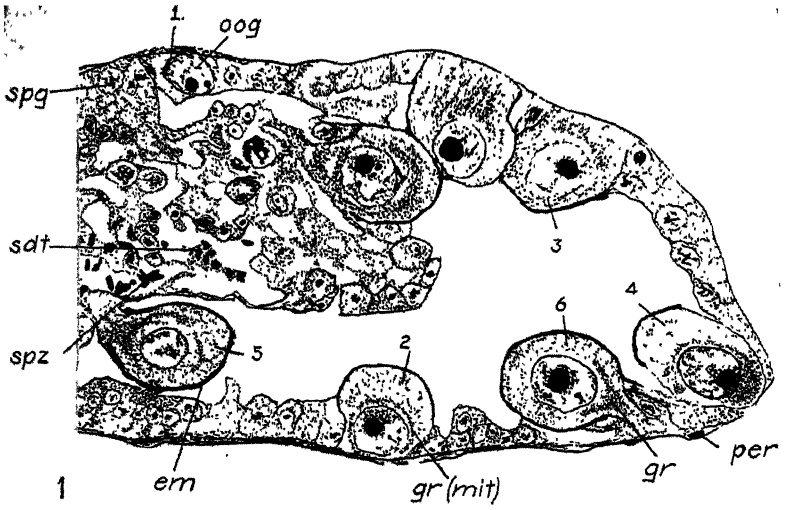


Fig. 1 Longitudinal section through a maturing gonad. Underlined numerals indicate successive stages in the growth of the oocytes. × 290.

Since this study deals with the germ cells, the cleavage in other regions was followed only so far as was necessary to determine the absence of any contribution to the formation of germ cells. The more general comparisons will not be made, since this has been done by Wilson ('92), Lillie ('95), Conklin ('97), Tannreuther ('15), and others. References to the literature will be made only as seems necessary in making my own account clear.

The ovum of Sphaerium, which is slightly oval, is oriented from the earliest stages (fig. 2). A membrane develops around the egg while it is still attached to the wall of the ovary (fig. 1, *em*). The point of attachment is marked by a large micropyle. A shift of cytoplasmic materials is indicated by the fact that the mitochondrial cloud, which is adjacent to the micropyle in the attached egg (fig. 1, no. 6, *gr.*), lies to one side of the micropyle in eggs free in the ovary

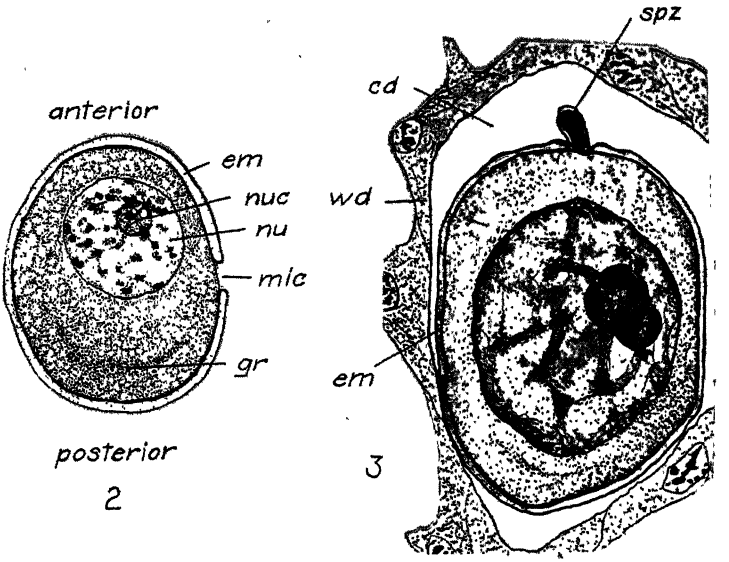
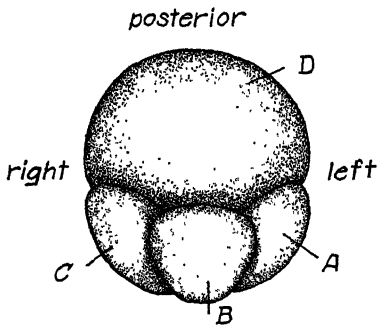


Fig. 2 Diagram showing orientation of the egg before fertilization.
Fig. 3 Sperm and ovum in the hermaphroditic duct. × 900.

(compare fig. 1, no. 6, and fig. 2). The nucleus shifts its position from the side of the egg opposite the micropyle to a place nearer the micropyle, but not adjacent to it. A line drawn through the nucleus and through the thickest and thinnest regions of the cytoplasm lying on opposite sides of the nucleus represents the future anteroposterior axis. Mitochondria seem to be the only storage material in the cytoplasm. A detailed account of the origin, distribution through cleavage, and significance of these mitochondria will appear

in another paper. The position of the nucleus, the micropyle, and the cloud of mitochondria establish the orientation of the egg with reference to the anteroposterior axis of the embryo. Dorsoventral orientation is not established until the second cleavage. Segmentation is holoblastic and unequal from the beginning.

The first cleavage spindle lies in the long axis of the egg and is nearer the animal pole. As a result, the egg divides into two cells of unequal size, *AB* and *CD*, at about the level of the polar bodies (figs. 6 and 15). Before the first cleavage



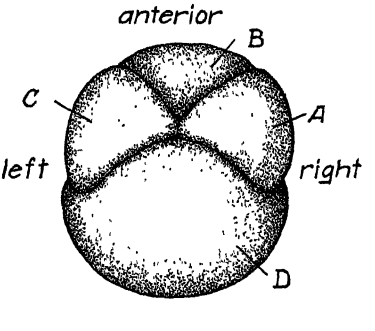


Fig. 4 Orientation of the four-cell stage as seen from above.
Fig. 5 Orientation of the four-cell stage as seen from below.

spindle forms, the chromosomes appear and the large vesiculated nucleolus becomes diffuse. At the same time, a clear area appears in the cytoplasm around the posterior side of the nucleus. When the nuclear membrane disintegrates and the spindle is formed, the nucleolus is no longer visible, but it reappears when the nucleus is reorganized in the daughter cells. The nuclei in *AB* and *CD* are spherical. The cytoplasm of *CD* is dense and finely granular, while that of *AB* is more hyaline. The cell membranes are distinct and the cells lie flattened together within the egg membrane (fig. 6). The two cells taken together approximate the shape of the mature egg.

The second cleavage is almost at right angles to the first and is well advanced in *AB* before there is any indication of spindle formation in *CD*. The result is a three-cell stage consisting of two small macromeres, *A* and *B*, of equal size, slightly to the left of the future midline of the body, and the large cell *CD*. The contact surfaces are flattened as they were in the two-cell stage (figs. 7 and 16).

After cleavage has been completed in *AB*, constriction occurs in *CD* and the four-cell stage results. The spindle is tilted (fig. 8) so that the cell *C* occupies a position on the cell *D*, as indicated in figure 17. On the future dorsal surface (fig. 4), the cells *B* and *D* are in contact, while *A* and *C* (fig. 5) are in contact for a shorter distance on the ventral surface. As seems to be the case in all eggs in which the greater mass of the four cells forms the ectoderm, the cross furrow resulting from this arrangement (fig. 4) is longer at the animal pole. In forms like Clepsine and Nereis the greater mass of the four cells becomes endoderm and the furrow is longer at the vegetal pole. Unlike the case of an egg having free development, the cross furrow is of little value as a landmark for orientation in Sphaerium.

Exact orientation of the four-cell stage is important for determination of the origin of regions that later develop (figs. 4 and 5). The cell *B* is anterior, *C* is on the future right side, *A* is on the left, and *D* is posterior. These cells are the future posterior ventral surface of the embryo, while the region of micromere production is the future dorsal surface. The future median plane passes through the cells *B* and *D*.

The cytoplasmic structure of the four-cell stage is much the same as in the preceding stages. The large cell *D* (fig. 9) is more nearly opaque and its cytoplasm more densely granular than the cytoplasm of the other three cells. The nucleus of *D* is slightly larger than the nucleus of *C*, which in turn is larger than the other two nuclei. The first indication of a blastula cavity appears in the four-cell stage (fig. 9).

First generation of micromeres. The production of micromeres occurs on the upper surface of the macromeres in a

somewhat irregular manner. After *C* has been cut off from *D*, it divides to produce *C* and *c.1*, thus forming a five-cell stage (fig. 18). This *c.1* is the first cell of the first generation of micromeres. Division in *C* is oblique. The micromere end of the spindle is uppermost and inclined toward the right, so that *c.1* lies between *C* and *D* on the upper surface. There are now four cells on the anterior upper surface of *D*. The second micromere of the first generation is produced by *D*. The spindle is tilted toward the right as it was in *C*, and as a result *d.1* lies on the surface in contact with *B*, *D*, *c.1*, and *D*. Cleavage in *D* usually begins before spindles form in *A* and *B*. *A* and *B* divide at about the same time and may complete their division to produce *a.1* and *b.1*, respectively, shortly after *d.1* is completely separated. The result is an eight-cell stage (fig. 21). Usually *d.1* has begun to divide before *a.1* and *b.1* have been cut off completely, so that a nine-cell stage results with the separation of *d.1* into *d.1.1* and *d.1.2*. The spindles in both *A* and *B* are inclined clockwise. The cell *a.1* lies between *A* and *B* and the cell *b.1* lies between *B* and *C* (fig. 23).

The internal structure of the micromeres is similar to that of the cells *A*, *B*, and *C*. The cytoplasm of the cell *D* is more coarsely granular and lacks the reticular appearance that is seen in the other cells after fixation. The nucleus in *D* is larger than those of the smaller cells. The egg membrane, which has been visible up to this time as a homogeneous cov-

Fig. 6 Section of the two-cell stage. × 430.

Fig. 7 Section of a three-cell stage. × 430.

Fig. 8 Reconstruction from three sections, showing the spindle that produces *C* and *D*. × 400.

Fig. 9 Section of a five-cell stage between the lines shown in figure 18. × 430.

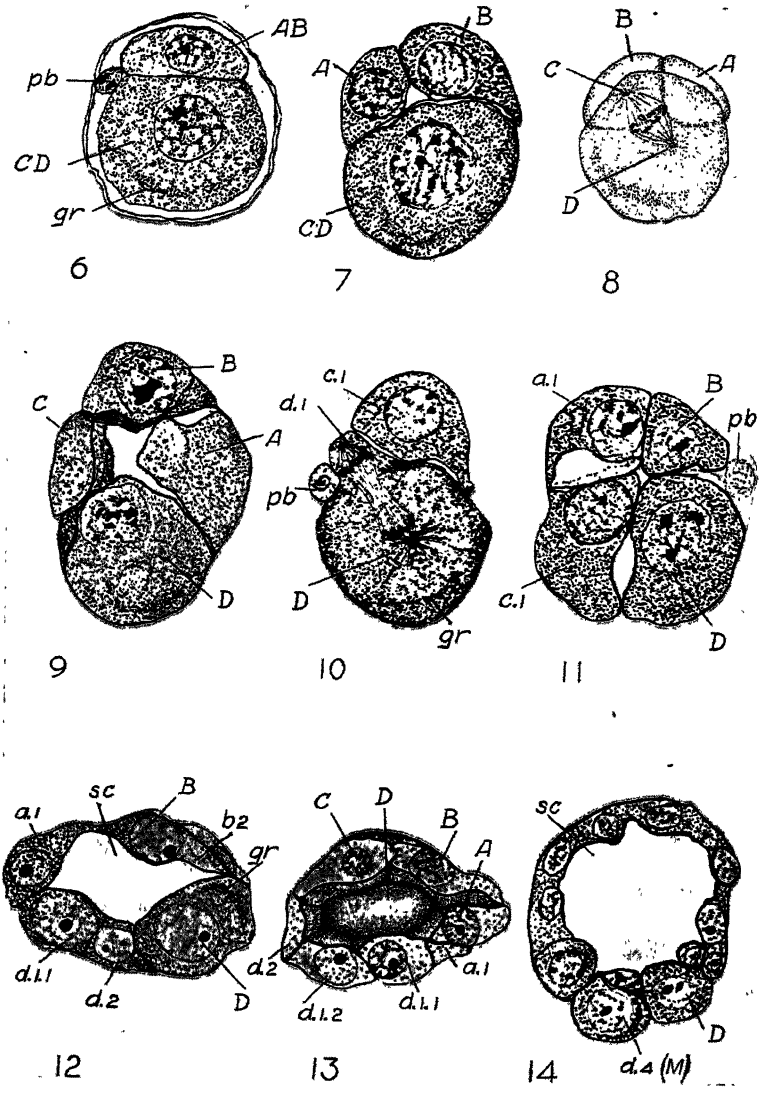
Fig. 10 Section through a five-cell stage, showing the spindle that produces *d.1*. × 430.

Fig. 11 Section of an eight-cell stage. × 430.

Fig. 12 Section of a twelve-cell stage at level 12 of figure 22. To show relative size of cells and segmentation cavity. × 430.

Fig. 13 Section at level 13 of figure 23. Half-schematic figure constructed from three sections. × 430.

Fig. 14 Section of thirty-two-cell stage, showing *D* and *M* on the surface before migration of *M* to the inside. × 430.



Figures 6 to 14

ering around the dividing cells, now begins to disappear. The blastula cavity is now more evident and lies within the upper half of the mass of cells. The unequal cleavage just described can be correlated with the fact that *CD* gives rise to cells that later produce the shell gland. The larger size of cell *D* can be correlated with the fact that it contains most of the future mesoderm and some of the endoderm. The cleavage is oblique, since the spindle in each of the macromeres inclines so that the micromeres are located to the right and above the cells that produce them.

Second and third generations of micromeres. Inequalities in the division rate in various regions result in some irregularity in the location of cells. This obscures the symmetry that is so clearly shown in the cleavage stages of animals that produce micromeres synchronously. While the division of *d.1* to form *d.1.1* and *d.1.2* progresses, the cell *D* gives off *d.2,* which is the first of the second generation of micromeres. Thus a ten-cell stage is formed (fig. 23). Division in *A* occasionally precedes division in *B,* and occurs at the time that *d.2* is being produced, so that an eleven-cell stage occurs.

The usual arrangement of cells, as shown in figure 21, is followed by cleavage in *A, B,* and *C,* forming a thirteen-cell stage (fig. 24). Division in *A* and *B* is always slightly in advance of that in *C,* thus producing a twelve-cell stage (fig. 22). The order of production of the second generation of micromeres is *d.2, a.2, b.2,* and *c.2.* The cell *d.2* is homologous

Fig. 15 Model of two-cell stage.

Fig. 16 Model of the three-cell stage. Position of the spindle shown in *D.*

Fig. 17 Model of the four-cell stage from the lower pole.

Fig. 18 Model of the five-cell stage from the upper pole.

Fig. 19 Model of the six-cell stage from the upper pole and slightly to the left.

Fig. 20 Model of a seven-cell stage, a case in which division of *d.1* occurs before division occurs in *A* and *B.*

Fig. 21 Model of the eight-cell stage from above and posterior. Cell *B* is not visible.

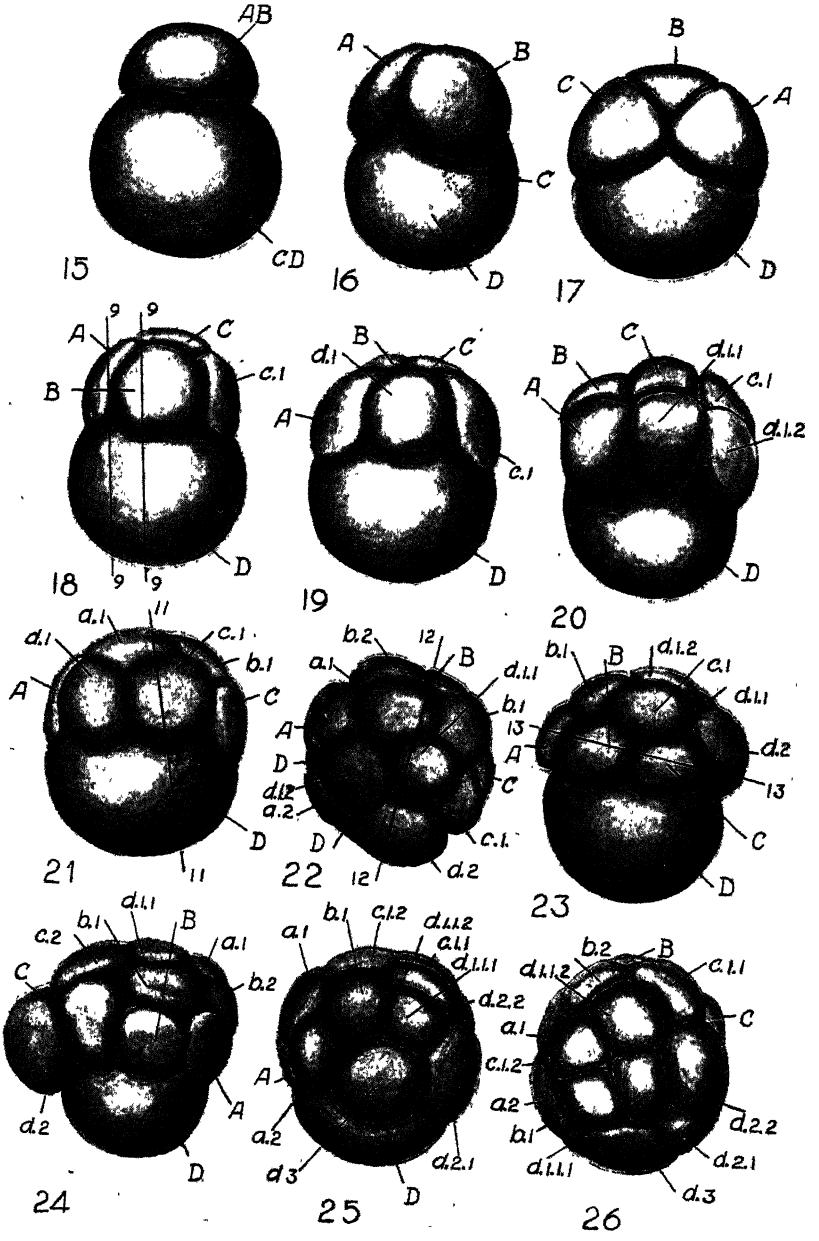
Fig. 22 Model of the twelve-cell stage from the upper pole.

Fig. 23 Model of the ten-cell stage from the side.

Fig. 24 Model of the thirteen-cell stage from the side.

Fig. 25 Model of the seventeen-cell stage from the side.

Fig. 26 Model of the seventeen-cell stage from the anterior end.



Figures 15 to 26

with the cell X of Bdellodrilus (Tannreuther, '15), two species of Nereis (Wilson, '92), Clepsine (Whitman, '78), and no doubt many other forms. Later it divides equally and gives rise, in all the forms in which its history has been followed, to the greater part of the ectoderm in the middle region of the embryo on the ventral side. I have not followed the cleavage of *d.2* past its third division, but, so far as I can determine, there is no connection between this cell and any portion of the mesoderm-forming cells. In addition to the cells resulting from the division of *d.2,* the cells produced by the subsequent divisions of *A* and *B,* or the cells *a.2* and *b.2,* contribute to the formation of the ectoderm, but in the anterior region of the body. Cells *a.2* and *b.2* probably give rise to certain cells that become the larval mesoblasts. These are designated by Ziegler ('85) as mesenchyme cells and later form the single-celled muscle fibers of the embryo. Their exact origin has not been determined. It seems certain that they have an entirely different origin from the principal mass of the mesoderm, because they are present before the definitive mesoderm masses appear in the blastula cavity. The formation of larval muscles from the descendant of 'Y' (or *a.2.2.1*) has been described in Unio by Lillie ('95). Stauffacher ('93) recognized these cells as ectodermal in origin and so confirmed the supposition of Ziegler ('85) that the mesenchyme cells are derived from ectoderm. This has also been described in other forms (compare Meisenheimer, '01 b).

The appearance of the nuclei and the relative size and position of the cells and segmentation cavity in the twelve-cell stage can be seen in figures 12 and 13. After the thirteen-cell stage, cleavage occurs in *d.2, d.1.1,* and *c.1,* while *D* gives rise to a third micromere, *d.3,* which is the largest so far produced. Although the cleavage of *d.3* was not followed past one division, it seems to lie in the region where the shell gland appears in later embryonic stages. Conklin ('97) finds that *d.2* gives rise to the shell gland in Crepidula. This is also true for Unio. It is possible I have misinterpreted its origin in Sphaerium, although the conclusion I have reached

seems to be justified by my observations. The result of these cleavages is a seventeen-cell stage (fig. 25). Cleavage in *C* results in the cell *c.2* being given off to the left, and *d.3* is given off to the right, opposite to the direction of *d.2*.

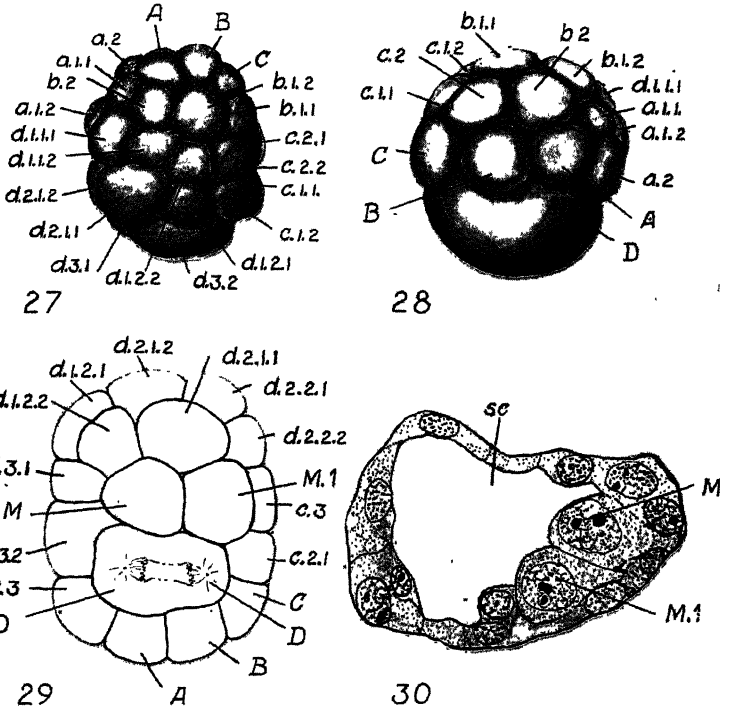


Fig. 27 Model of the twenty-four-cell stage from the upper pole.

Fig. 28 Model of the seventeen-cell stage from the upper pole.

Fig. 29 Diagram of the arrangement of cells about the posterior end just after division of *d4*(*M*).

Fig. 30 Cross-section of a late blastula just after *M* and *M.1* have reached the interior. × 250.

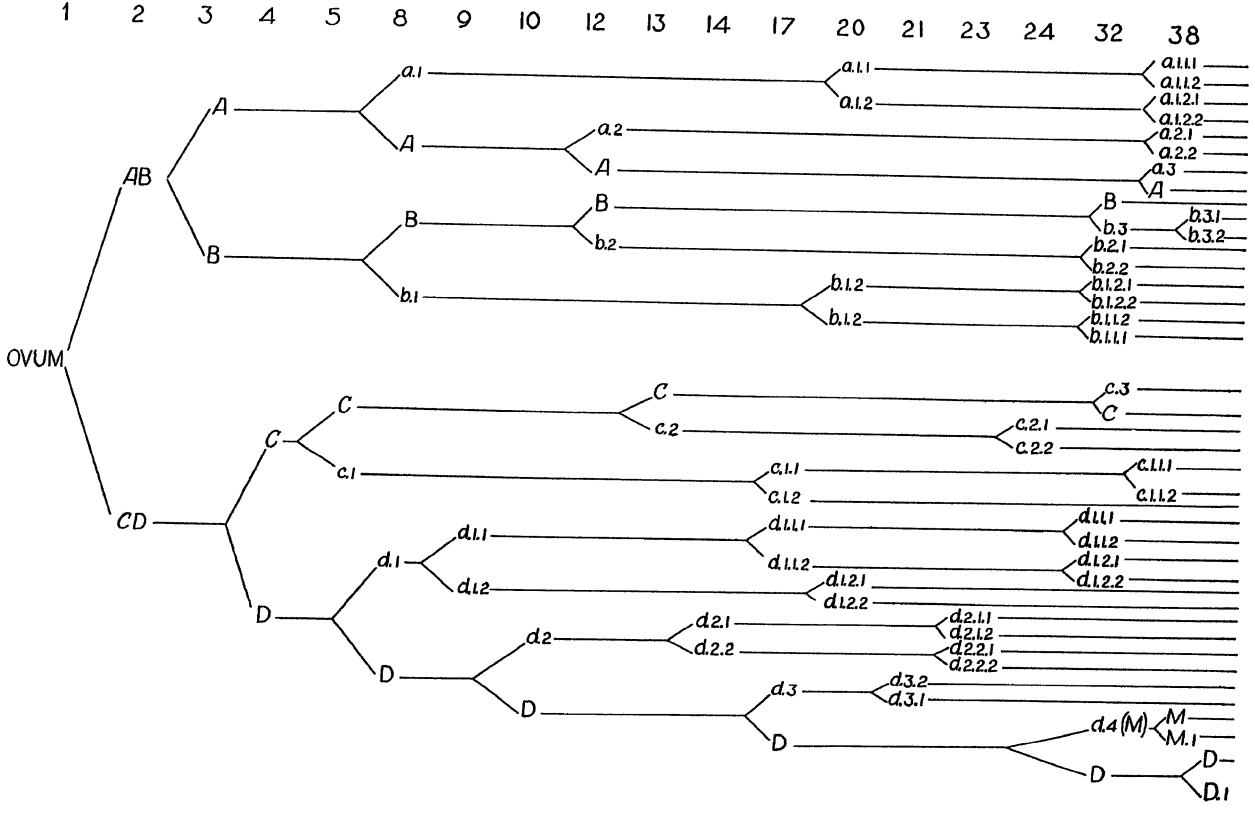
Cleavage in *a.1*, *b.1*, and *d.1.2* is followed by cleavage in *d.3* to produce a twenty-one-cell stage. Cleavage in *d.2.1*, *d.2.2*, and *c.2* produced a twenty-four-cell stage (fig. 27) before *b.3* and *c.3* are formed. The third generation of micromeres is given off to the right, opposite to the direction of the second generation.

Mesoderm formation and segregation. At about the twenty-four-cell stage *D* begins to divide equally to produce *d.4*, but the completion of this division is not accomplished until about the thirty-two cell stage. This stage, however, is merely a coincidence in the number of cells, since the smaller cells of the upper hemisphere have never divided synchronously. The fourth division of *D* is almost at right angles to the earliest division. With the increased number and changing position of the cells, there has also occurred an increase in the alternate change in the direction of the spindles in *D* until this last one is slightly more than 90° from the direction of the first cleavage spindle in the egg. The cell *d.4* (fig. 14), which is the mesoderm-forming cell, is now called *M*. From this time on the cell *D* contributes only to endoderm formation. Hence the production of *d.4* finally separates the mesodermal- and endodermal-forming capacities which were previously carried in *D*.

There is one equal division in *M*, forming *M* and *M.1* (fig. 29), and at about the same time there is an equal division in *D*, with the result that there are four large cells on the posterior surface of the embryo which are the products of the most recent divisions of *D*. In addition to these four cells, the cells *A*, *B*, and *C* on the posterior surface also form part of the endoderm. The number of cells present in the embryo at the time *d.4* is produced is somewhat variable in Sphaerium and differs greatly in different species. In Bdellodrilus it occurs at the twenty-four-cell stage, in Unio at the thirty-two-cell stage, in Nereis at the thirty-eight-cell stage, in Dinophilus at the forty-five-cell stage. In Crepidula *d.4* is produced at the twenty-nine-cell stage, although final separation of all the endoderm-forming material from *d.4* does not occur until the sixty-five-cell stage. The number of cells present when the primary mesoblast '*M*' (*d.4*) is produced cannot always be correlated with a precocious development in certain regions as was formerly thought (compare Lillie, '95, p. 77).

The statement of Stauffacher ('93) that the mesoderm is paired from the outset is true, if he means it is not mesoderm

TABLE 1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2
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17
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23
24
32
38
OVUM
AB
CD
A
B
C
D
a.1
a.2
a.3
b.1
b.2
b.3
c.1
c.2
c.3
d.1
d.2
d.3
d.4 (M)
M
M.1
D
D.1
a.1.1
a.1.2
a.1.2.1
a.1.2.2
a.2.1
a.2.2
b.3.1
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until it is on the inside. His statement may also be due to a different interpretation of the cells that lie in the region of the posterior end. According to my observations, the unpaired cell *M*, which lies on the surface of the embryo, is the parent of all mesodermal elements except the larval mesoblast. The macromere *D*, which is the cell *Ma.* in Stauffacher's description, does divide into two parts as shown in figure 29, but the macromere *ma.2*, which he shows in his figure 28, is no doubt *M.1*, and the cell he labels as *ma.1* seems to be *M*. The cell he has labeled *um.1* in his figure 28 is the second division of *M*.

With the division of *M* to form *M.1* and *M*. (fig. 29), we find the paired mesoblasts on the posterior surface of the embryo adjacent to the cell *D*, which is dividing to form *D* and *D.1*. Both of these mesoderm-forming cells now begin to sink below the surface. Just before this migration each begins to bud off a smaller cell into the blastocoele. These new cells are on the inner surface of the parent cell, but lie in contact with the ectoderm when the division is completed. As the large cells *M* and *M.1* migrate inward, the cells on the surface divide and close the place that has been vacated (figs. 30 and 31). With the completion of this process there are four mesoderm cells, derived from the original unpaired cell *M* or *d.4*, lying within the cavity in contact with the posterior surface cells. There are also four cells on the surface that result from the two most recent divisions of *D*. The production of the smaller mesoderm cells is in such a direction that they lie next to the blastula wall. The division of the two smaller cells interposes another cell between the large cells *M* and *M.1* and the blastula wall. A second generation of small mesoderm cells is given off by *M* and *M.1.1.2*. These also lie next to the blastula wall and are the cells *m.2* and *m.1.2.1*. The result is a mass of small cells next to the blastula wall on either side, with one large cell on the inner surface of each mass. The cells *D* and *D.1* divide at about the same time as the first division of *M* and *m.1*. A third generation of small mesoderm cells is produced by *M* and *M.1.2.2*, form-

ing the cells *m.1.2.2.1* and *m.3*. This is the last unequal division of the primary mesoblasts. There are then two large cells, one on either side, and between these and the ectoderm are the smaller cells of similar origin. Since the smaller cells are produced between the larger cells and the blastula wall, they tend to push the large cells farther into the cavity. A few larval mesoblasts are also present (fig. 32, *mch.*), but they are of different origin from the two mesodermal masses

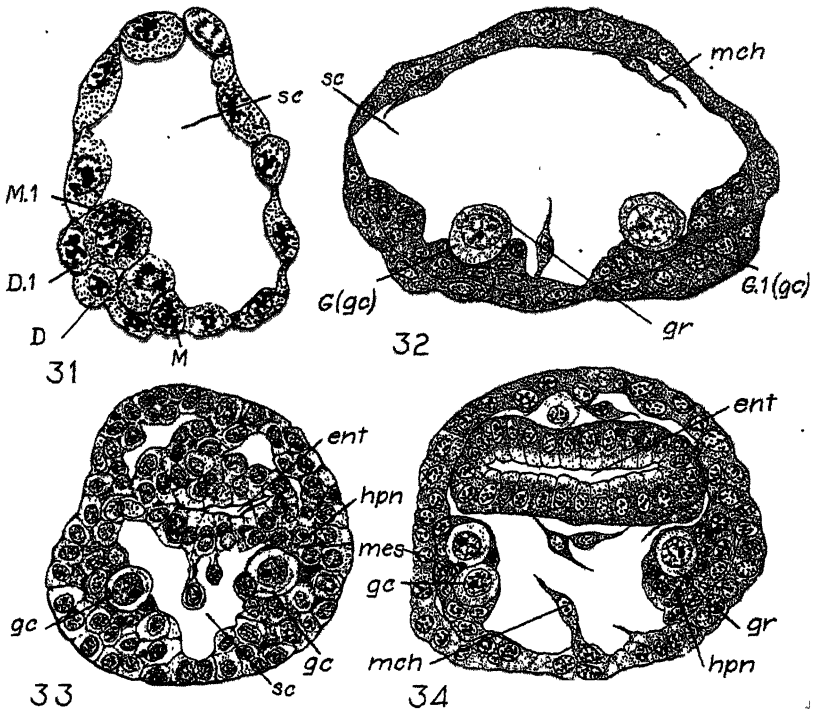


Fig. 31 Section through a blastula in which the migration of *M* and *M.1* to the interior is complete. × 215.

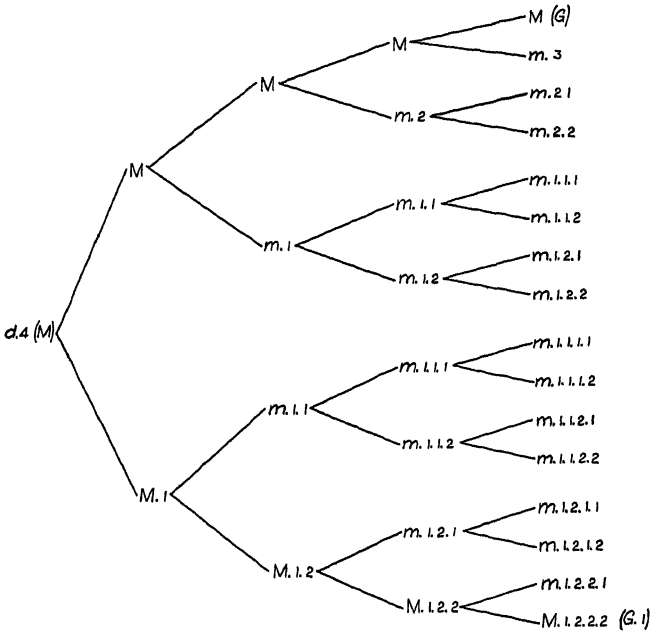
Fig. 32 Transverse section through a late blastula, showing the meodermal masses on each side ventrally, and the two original primordial germ cells. × 300.

Fig. 33 Transverse section through an early gastrula, showing the location of the germ cells and mesodermal masses. Only one germ cell shows on either side. × 250.

Fig. 34 Transverse section through a late gastrula, showing location of germ cells. × 250.

derived from the primary mesoblasts *M* and *M.1*. *The large cells, M and M.1.2.2.2 (G and G.1 of fig. 32) may now be called germ cells, since they ultimately produce the definitive cells of the germ gland and retain their characteristics throughout the remainder of the embryonic development until they reach their place in the gonad. They are, moreover, the only cells that form the definitive germ cells and all the*

TABLE 2



products of their subsequent divisions appear to be potential germ cells. Each of these cells *G* and *G.1* (fig. 32) divides once, equally, and at about the same time, along with the irregular division of other cells in the mesodermal masses. The daughter cells of *G* and *G.1* then remain inactive for a relatively long period. Stauffacher ('93), in his figure 32, shows a large cell in the mesoderm, as it appears on one side of the embryo, that has all the characteristics of a primordial germ cell, though he does not call it that.

In the mitoses that result in the setting aside of the primordial germ cells there is no evident difference in the nuclei of the larger and smaller cells except in size. Favorable material for the study of chromosomes has not been obtained. So far as I have been able to determine in Sphaerium, no elimination of chromatin occurs as in Ascaris (Boveri, '92) and Miastor (Kahle, '08, and Hegner, '12). Elimination of chromatin is not peculiar to primordial germ cells, as shown by Rhode ('11) and Wilson ('25). In Sphaerium, however, certain chromatin material always seems to go on the equatorial plate before the remainder. During the early anaphase (figs. 50 and 51) of mitosis there are also four of these masses ahead of the remainder of the chromatin in its passage toward the poles. I have no suggestion as to the reason for this. So far as I can determine, no chromatin is discarded in any mitosis.

During the migration of *M* and *M.1* the endoderm begins to be formed. The cell *D* divides equally and all its descendants become endoderm. Gastrulation occurs by a true invagination, as described by Lankester ('75 a and b, '76). At first the archenteron is short and extends into the segmentation cavity between the paired mesodermal masses and serves to separate them farther. Differentiation of the endoderm to a functional state, which has been described by Ziegler ('85) and others, need not be considered here. A comparison of the various cases in which the endoderm has different modes of origin is made by Tannreuther ('15). The amount of endoderm derived from *M* varies from cases like Crepidula, in which *M* gives rise to more endoderm than mesoderm, to the other extreme in which *M* contributes nothing to the endoderm, as in Bdellodrilus. In Sphaerium, also, *M* contributes nothing to the endoderm.

Discussion. Under normal conditions of development cleavage in Sphaerium is determinate. Both embryonic and adult structures are traceable from definite regions of the unsegmented egg. Orientation of the future embryo is partially forecast by the relative position of the nucleus, the

micropyle, and a cloud of granular mitochondria. Although cleavage thus separates the various regions of the egg having different capacities for differentiation, it is not believed that egg cytoplasm is passed unchanged through successive generations of cells. A detailed comparison of cleavage in the molluscs, annelids, and flatworms is not necessary in this discussion, but certain points must be considered.

The general plan of cleavage in Sphaerium is similar to that in many other forms. Three successive divisions separate the ectoderm from the basal cells. The fourth cleavage of the macromere *D* separates the mesoderm except for the larval mesoblasts. The remaining cells form the endoderm. In the molluscs, except the cephalopods and Lymnaea, in annelids, turbellaria, and nemertines, this mosaic development of the egg is of the oblique or spiral type with the first and third generations of micromeres arising dexiotropically and the second and fourth generations arising leiotropically, and this relationship is constant regardless of the wide differences in the size of the basal cells occurring in the various forms. It is not necessary to discuss the complicated problems concerned in the arrangement of cells during these cleavage stages except to point out that the rate of growth, size of the cells, their content and physical nature are all factors which are concerned in the positions that the cells occupy and in the form of the mass as a whole. Despite the development in a brood pouch, cleavage in Sphaerium is similar to the type commonly found in most of the annelids and molluscs that have been carefully studied.

That the germ cells are mesodermal in origin has long been known for Sphaerium, but their definite history through the cleavage stages has not been followed. The formation of the mesoderm from the fourth generation of micromeres arising from the cell *D* of the four-cell stage has been shown to occur in Clepsine (Whitman, '78); two species of Nereis, Spio, Polymnia, and Aricia (Wilson, '92); Fiona (Casteel, '04); Amphitrite (Mead, '97); Bdellodrilus (Tannreuther, '15); Thalassema (Torrey, '03); Lumbricus (Kleinenberg, '79, and

Wilson, '90); Capitella (Eisig, '98); Podarke (Treadwell, '01); Arenicola and Sternapsis (Child, '00); Neretinea (Blockmann, '82); Planorbis (Holmes, '00); Unio (Lillie, '95); Argobuccinium (Philpott, '25); Physa (Wierzejski, '05); Crepidula (Conklin, '97); Dinophilus (Nelson, '04); Aplysia (Carazzi, '00); Umbrella (Heymons, '93); Sphaerium, and other forms and thus appears the most common, if not the universal, mode of origin for this germ layer. In the polyclad Discocoelis, according to Lang ('84), the mesoderm is derived from the ectomeres. Mead ('97), because of the origin and behavior of another cell in Discocoelis, believes it to be the mesoderm cell. This has not been verified for Discocoelis, although Surface ('07) finds that in Planocera the cell *4d* produces the mesoderm and all of the endoderm, since the macromeres degenerate. Mead ('97) states that the cell *M* belongs to the 'ideal' sixty-four-cell stage in Amphitrite, and believes this is true for other forms. This is not the case. In Sphaerium and in Unio, *M* is produced at about the thirty-two-cell stage, in Dinophilis and Arbobuccinium at the twenty-nine-cell stage, and in Dreissensia at the forty-five-cell stage.

Regardless of the time of its production and its relative size as compared to *D,* the cell *M* always divides equally. In most cases the division that produces *M* is very unequal (compare Bdellodrilus, Dinophilus, Crepidula, Amphitrite). In Sphaerium this division is about equal. Equal division in *M* constitutes the first bilateral division in the posterior part of the embryo in all forms having spiral cleavage except in Planocera, where there is one unequal division of *4d* before equal division begins. In some forms equal division is delayed as in Dinophilus (Nelson, '04). In Sphaerium it occurs very early. Further comparisons are unnecessary. The important point is the bilateral division of *M* in all cases cited except Planocera.

The kind of division occurring in the paired mesoderm-forming cells is also strikingly similar in the various forms studied. With the exception of Clymenella, in which the first daughter cells of the pair are the same size as the parent,

a teloblastic cleavage occurs in these cells that results in the production of the mesoderm bands. These bands were thought to be ectodermal in origin by Salensky ('82) and Kleinenberg ('79 and '85). Wilson ('90) demonstrated their true origin. The number of cells produced by teloblastic division is variable. In Sphaerium three divisions in these cells along with division of the daughter cells results in the establishment of two irregular masses of mesoderm. The absence of definite germ bands such as occur in annelids is no doubt correlated with differences in adult form between annelids and molluscs. Division of the paired mesoderm cells may begin before or after they migrate to the interior. In Sphaerium the production of small mesoderm cells begins before and ends after the large cells have reached the inside. In Podarke, Treadwell ('01) finds that migration occurs before *M* and *M.1* divide.

The major portion of the mesoderm in a majority of forms is shown to be derived from the cell *d.4* (*M*) of the cleavage stages, although mesoderm of different origin is present in most species in the form of larval mesoblasts. In all cases the behavior of these two kinds of mesoderm is different. Larval mesoblasts divide irregularly and are mesenchymatous, while division in the primary mesoblasts is teloblastic and their derivatives adhere to form the germ bands. Lillie ('95) states that the larval mesoblasts provide the contractile elements used in the early stages of development. They are therefore transitory. The exact origin of the larval meso-

Fig. 35 Oblique transverse section through an early trochophore stage, showing the two germ cells on one side only. × 258.

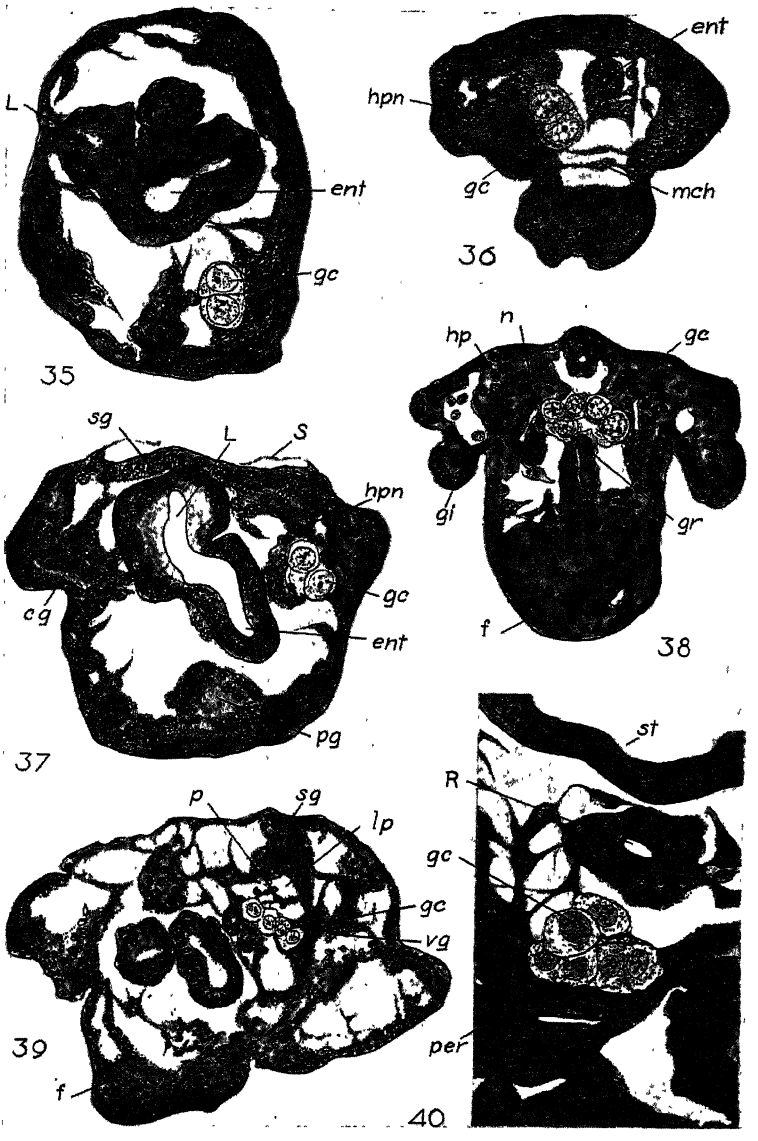
Fig. 36 Oblique transverse section of a slightly older trochophore than is shown in figure 35. The relation of the germ cells to the anlage, pericardium, and nephridium is shown. × 215.

Fig. 37 Longitudinal section through an early-embryo stage, to show the relative position of the germ cells and the archenteron. × 215.

Fig. 38 Transverse section of an embryo at the end of the inactive period of the germ cells. × 215.

Fig. 39 Longitudinal section through an embryo, showing the formation of the right gonad. × 100.

Fig. 40 The gonad region of a slightly older embryo, showing the difference in appearance of the germ cells and adjacent cells. Oblique section. × 430.



Figures 35 to 40

blasts was not determined for Sphaerium. They probably come from cells in the anterior region of the embryo, since a few scattered cells are present in the region of the descendants of the second generation of micromeres anteriorly. None of it is formed from *M*. In Unio, Sphaerium, and Bdellodrilus *M* is all primary mesoderm, while in Thalassema, Crepidula, Podarke, Physa, and others it contributes in varying amount to endoderm formation also. The remainder of the mesoderm is formed in different ways in various species. In Thalassema the first and third generations of micromeres in the *a, b,* and *c* quadrants contribute; in Unio *a.2* is the only other cell that contributes; in Crepidula, *a.2, b.2,* and *c.2;* in Podarke, *a.3, b.3,* and *c.3;* in Physa *b.3* and *c.3* contribute. Further comparisons could be made, but would serve only to show that forms can be found in which almost any combination of the first, second, third, and fourth generations of micromeres contribute to the mesoderm as a whole. The exact origin of the larval mesoblasts was not determined in Sphaerium, because it is evident that these cells contribute nothing to the formation of germ cells.

Period of inactivity of the germ cells

Description and location. At the time of gastrulation (figs. 32 and 33) two masses of cells, in contact with the body wall and one on either side near the posterior end, constitute the major portion of the mesoderm. In each mass are two cells (fig. 33, *gc*) that have been designated as primordial germ cells, since they are structurally different from other cells and ultimately form the definitive sex cells in the gonad. Meisenheimer ('01 a, p. 426) describes the production of germ cells as the first differentiation of this mass and shows by a comparative table, including Paludina, Cyclas (Sphaerium), Dreissensia, and Limax, that the heart, nephridium, pericardium, and germ cells all develop from this mass of undifferentiated cells. The order of production of these structures varies in the different genera. In Sphaerium the order is primordial germ cells, nephridia, and lastly the heart and pericardium.

These symmetrically rounded masses of mesoderm in Sphaerium are composed in part of cells probably derived from blastomeres in the anterior part of the embryo, but mostly derived from the cell *d.4*, or *M* of the cleavage stages. Although the mesoderm cells considered collectively have a double origin, no attempt will be made to distinguish between the two kinds in the remainder of the discussion. It has been pointed out that the germ cells are derived from the primary mesoblasts *M* and *M.1*. The larval mesoblasts contribute nothing to their formation.

The four primordial germ cells (figs. 34, 35, and 36, *gc*) appear never to give rise to anything but potential germ cells and can easily be distinguished by their peculiar structure and appearance throughout later stages of development. As shown in these figures, these primordial germ cells are the largest in the embryo. Their nuclei are much larger than those of other cells and are clear except for the nucleolus and fine strands of chromatin. Their cytoplasm is somewhat clearer than that of the other cells and is smaller in proportion to the nucleus as compared with other cells. The germ cells retain this characteristic appearance during the intervening stages until they begin to increase in number as the definitive gonad is formed. There is no difficulty in recognizing them at any time.

Zeigler ('85) recognized that certain cells in this mass of mesoderm were germ cells. His figure 6 on plate XXVII shows a cell, *M.1*, that is similar to the larger cell shown in figure 32 by Stauffacher ('93). Zeigler ('85) also shows the germ cells of one side of the embryo after gastrulation in his figure 12 c, and in a later stage in his figure 19, both of plate XXVII.

Changes in location. After their first appearance in the mesoderm, the number of the primordial germ cells remains constant for a time, although their position changes. This is designated as the period of inactivity, because there is no change in appearance and because the changes in position are accomplished by factors outside the germ cells. During

this time there is rapid proliferation of cells in other regions, but the four primordial germ cells remain as two cells on each side of the embryo without further divisions until they resume their activity after reaching the place where the gonad is formed. The differentiation of the mesoderm is described by Meisenheimer ('01 a).

Four factors may conceivably play a part in this change in location: invagination; proliferation of cells in other regions and the consequent increase in size and change of shape of the embryo; movements of the embryo and of the contractile cells in particular; movements of the parent animal in which the embryo is developing. The first two factors would seem most important, the third probably would not exert any great influence in changing the location of the germ cells, while the last would be of little if any importance, since the shape of the embryo is maintained regardless of the disposition of parts in the parent.

With the ingrowth of the endoderm of the posterior midline, the two masses of mesoderm containing the germ cells are spread apart and pressed against the body wall. The subsequent elongation of the gut and the general elongation of the embryo consequent to invagination tend also to change the position of the mesodermal masses by moving them farther forward in the embryo. Proliferation of the mesodermal cells between the germ cells and the body wall carries the germ cells inward and increases the size of the remainder of the undifferentiated mesoderm (figs. 32 to 36). Rapid growth in the region of the foot and the sides of the body carries the body wall ventrally, so that the germ cells become more dorsally located (fig. 36).

At the beginning of the differentiation of the nephridia the germ cells lie on either side of the embryo at the lower margin of a mass that is forming the nephridia, but has not yet begun to form the pericardium. The nephridial masses elongate and become detached from the remainder and form the nephridial tubes, while the germ cells remain attached to the undifferentiated remainder of the mesoderm. The cavity that

now appears in the lower portion of the mesoderm above the germ cells is the lower pericardial cavity. The germ cells are attached to the ventral surface of its ventral wall. They begin division to form the cells of the definitive gonad during the formation of the upper pericardial cavity and the heart. Thus the period of inactivity is brought to an end and the final cycle of germ-cell development begins.

A detailed account of the changes occurring in the other mesoderm during the period of inactivity of the germ cells is given by Meisenheimer ('01 a) for Sphaerium. Zeigler ('85) recognized that the germ cells are finally separated from the lower pericardial wall, but gives a very meager description of the process.

Formation of the gonad and its regionation into male and female portions

In the early embryo. Differentiation of the kidney, pericardium, and the heart is not completed in Sphaerium until the latter part of the early-embryo stage. Before this time the germ cells have begun to increase in number (figs. 37 and 38). Thus there come to be two groups of germ cells attached to the ventral side of the differentiating pericardium and meeting in the midline (fig. 38). A thin layer of flattened cells surrounds the germ cells and holds them in shape as two bean-shaped, solid masses with the inner surfaces in contact. Proliferation of the germ cells is slow. Mitotic figures are rare as compared to the large number of actively dividing cells in the regions of gill formation and in the structures developing on the ventral side.

Both masses of germ cells increase in size. Since the body width increases more slowly than body length and depth, the gonads (figs. 39 to 42) find less resistance to growth longitudinally and ventrally. Elongation of the gonad occurs anteriorly until it is almost in contact with the liver, which in the meantime has been developing its secondary lobes and is extending backward toward the nephridia and gonads.

The number of cells on each side in the gonad increases from two to about twelve or fourteen during this period of the early embryo. Counts show approximately the same number on each side at any given time (figs. 42 to 44). It is possible that some of the cells were counted twice and that some were not counted at all, but the method of making camera-lucida drawings on transparent paper and then counting from these drawings is fairly accurate. The greatest variation in number on each side was two cells. Each of the new germ cells is derived from a cell like itself, already in existence, and never from somatic cells. The large size and peculiar structure of the germ cells make them easily distinguishable from the somatic cells of the peritoneum immediately surrounding the gonad. In the peritoneal cells (fig. 43, *per*) the nuclei are oval, with the greater diameter about twice the shorter. The nuclei of the germ cells are larger and spherical. This peritoneal covering of the gonad is also mesodermal in origin and its function is purely one of support. All the cells within the gonad at this time are germ cells, and even a superficial examination of cells in other regions of the body would not permit them to be mistaken for germ cells. No potential germ cells are found elsewhere in the embryo in Sphaerium. This differs from the condition in the vertebrates. Witschi ('21 and '22) finds indifferent cells, outside the gonad, that later undergo metamorphosis into typical germ cells. The same has been described for other forms (compare Firket, '12; Hargitt, '26, and Hann, '26).

Fig. 41 Transverse section through an older late embryo, showing the location of the gonad and its relation to other structures after the differentiation of the nephridium, pericardium, and heart. × 100.

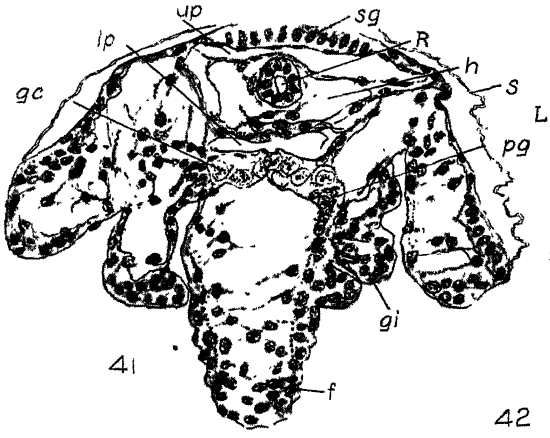
Fig. 42 Transverse section through a late-embryo stage, showing location of the gonad. × 50.

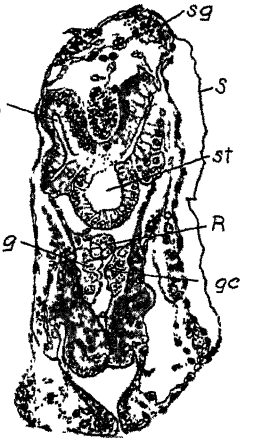
Fig. 43 Region of the gonad of figure 42, under higher magnification. × 200.

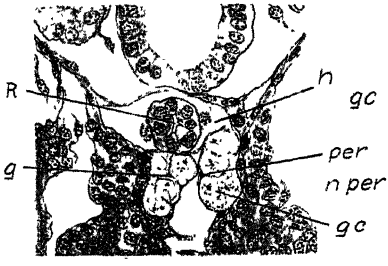
Fig. 44 Section of the gonad region of a slightly younger late-embryo stage than that shown in figure 43. × 200.

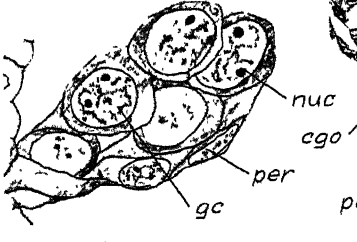
Fig. 45 Section of the right gonad of a slightly younger embryo than that shown in figure 44. Five of the eight primordial germ cells present are shown. × 650.

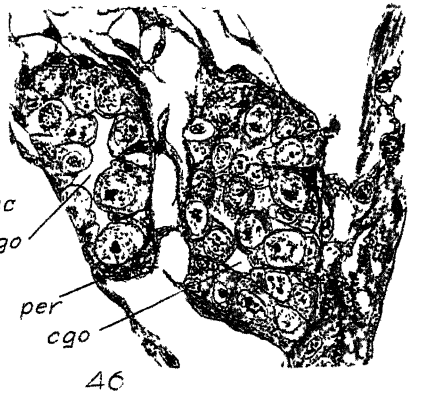
Fig. 46 Oblique frontal section of the gonad region of a very late-embryo stage. The cavity of the gonad is being formed. × 300.











Figures 41 to 46

In the late embryo. The best distinction that can be made between the early embryo and the late embryo is to characterize the former stage as a period of organ formation, and the latter stage as one of development of already formed organs to a state comparable with their condition in the adult. The history of the gonad in the late embryo as thus defined is principally a continuation of the early embryonic history, since the gonad is being formed early in the stages preceding the late embryo and before all the other organs have been formed. Its history in the later embryonic stages is characterized chiefly by increase in the number of primordial germ cells and by changes in size and shape. The new features of this period are the formation of the ducts and the separation of the male and the female region.

During early development the gonad (figs. 42 and 45) was a solid mass of germ cells surrounded by peritoneal cells lying on either side of the body next to the place of formation of gills and pedal ganglion. At this time both sides are the same size and shape. As the length increases the anterior ends of the gonads become separated farther and farther.

The originally solid gonad on each side develops a cavity in the center (fig. 46, *cgo*) that increases in size as the gonad grows. This cavity is carried posteriorly through the wall of the gonad by a spreading apart of the adjacent cells to a point where it empties into the common cloacal chamber near the opening from the nephridium. The mass of cells posterior to the gonad increases posteriorly until the wall of the cloacal chamber is reached. The opening appears in the anterior end and proceeds slowly toward the posterior end, reaching completion about the time the embryos are extruded from the parent. The elongation of the gonad produces two short club-shaped structures with their posterior thicker portions in contact, while their anterior thinner ends spread apart slightly as they near the liver in front. Small evaginations appear in the anterior wall of each gonad as it continues to grow forward. These shallow pockets deepen and become more definitely separated from one another to form

the lobes of the male region. The male and the female regions of the gonad are closely associated in the embryo, and complete separation of these two regions does not occur until the animal is older and has begun to produce mature spermatozoa and ova. The connection between the male and female portions of the gonad is the original cavity of the gonad, and as growth proceeds this cavity becomes attenuated to form the tube that carries the male sex products to the female

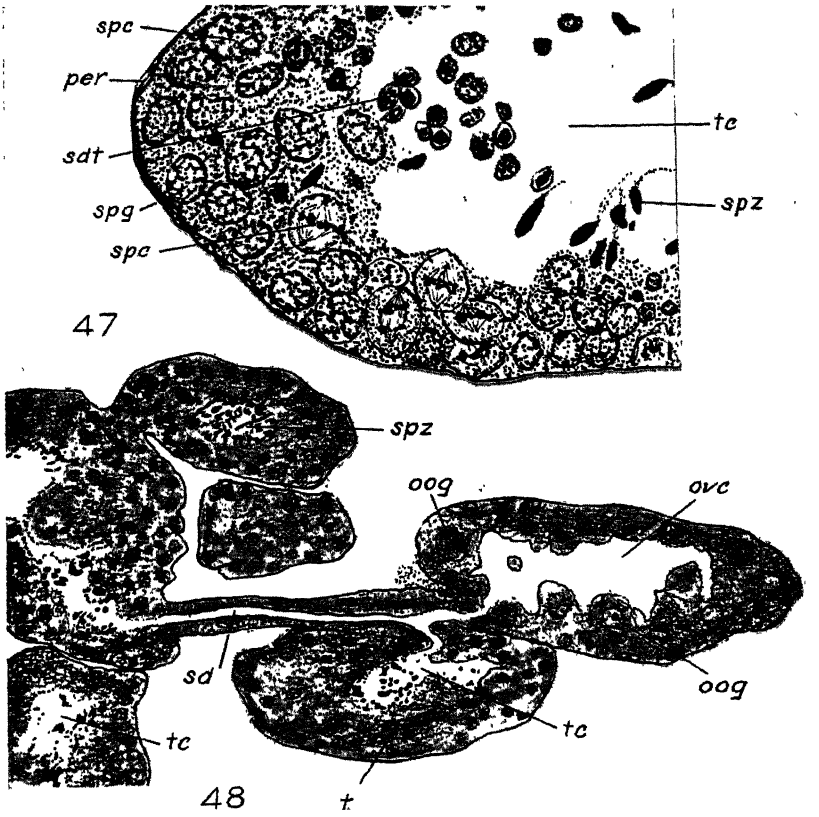


Fig. 47 A portion of one lobe of the testis of a mature specimen, showing regionation of cells in maturation. × 215.

Fig. 48 Longitudinal section through part of the gonad of a mature animal, to show the relation of the sperm duct of the testis and ovary.

region, from which they reach the cloacal chamber by way of the larger hermaphroditic duct. The female portion is not lobed, but consists merely of the enlarged posterior portion of the gland from which the hermaphroditic duct emerges (compare figs. 46 and 48).

Maturation

In the examination of successive stages after hatching there is no evidence of maturation in my material until the animals have reached a length of 2.5 mm. Stauffacher ('93) described the cytoplasmic growth of the oocyte in Sphaerium, but not the nuclear changes. I have found no account of the changes in the male sex cells during maturation. My observations confirm those of Stauffacher ('93), except that I have not observed oogonia or oocytes contributing to the storage of material in the growing oocyte, as shown in his figures 10a, 10b, and 10c of plate XI. While my material does not permit me to trace the details of meiosis, young oocytes show that the early stages are occurring during the very early growth period. The diffuse chromatin granules condense into leptotene threads (compare Winniwarter, '00), and in older cells these are condensed into rounded masses that occur in pairs. Evidently the synaptic stage occurs early during growth. The material as prepared does not permit counting of the chromosomes. These are always clumped on the spindle during the metaphase and anaphase stages, so that the number cannot be determined. It is presumed that both meiotic divisions occur after the egg is set free from the wall of the ovary, since no polar bodies are found before the eggs reach the brood pouches. I have never found either of the meiotic divisions in the egg.

In mature animals all stages of spermatogenesis are to be found in the lobed male region of the gonad throughout the year. Spermatogonia are indistinguishable from the primordial germ cells in the late-embryo stages (fig. 47, *spg*). They are peripherally located in the testis, while the first and second spermatocytes (fig. 7, *spc*) are nearer the cavity and the

spermatids (fig. 47, *sdt*) lie next to the cavity. The male region of the gonad is more favorable than the female region, but still does not show sharply defined chromosomes. Counts were made of the late prophase of the first spermatocytes and the numbers vary from sixty-eight to ninety-eight. Second spermatocyte prophases show slightly more individuality of the chromosomes, but here again counts gave varying results, ranging from thirty-two to forty-six. The chromosomes go on the spindle irregularly. Four of them always reach the equatorial position first. These four seem to have no permanent relation to the others. During the anaphase also (figs. 50 and 51) four chromosomes are always in advance of the others as they pass toward the poles. It is presumed that these result from divisions in the four that were first to arrive on the equatorial plate, although this could not be determined, since all the chromosomes are of about the same size and shape and their outlines are hazy. The chromosomes could not be counted in somatic cells of any body region. The chromosomes of all the cells are small and clumped on the mitotic spindle. This behavior of four chromosomes cannot be correlated with sex differences in the primordial germ cells, because it occurs also in somatic cells. It has been observed in the gills, the lining of the gut, cleavage stages, brood-pouch cells with multipolar spindles, and elsewhere in the animal (compare figs. 50 to 53).

The first meiotic division seemed to be reductional, at least for the majority of the tetrads. Secondary spermatocytes occur in pairs in the prophase and each contains approximately half the number of chromosomes present in the prophase of the first spermatocyte. In the late telophase of the second meiotic division the chromosomes are closely clumped at the ends of the spindle. When the two cells separate, spermioteleosis begins (fig. 47). The spermatids always occur in quartets (fig. 47, *sdt*) immediately after the second meiotic division. The periphery of the early spermatid nucleus stains deeply, while the central portion is clearer and stains faintly. Very little cytoplasm is present. The material was not fixed

to demonstrate the Golgi bodies, hence no statement can be made concerning their behavior. No visible structures can be interpreted as acroblasts or acrosomes, either in the early or late stages of spermiogenesis. Very fine granular mitochondria are present. These become condensed in the elongating spermatid to form a nebenkern consisting of a variable number (two to six) of relatively large granules that stain with acid fuchsin. Their history was not followed. Centrioles are not visible in either spermatids or spermatozoa, although they are evident during the metaphase and early anaphase of mitosis. The mature spermatozoon is fusiform, with an elongated head.[1] The middle piece shows a deeply staining area adjacent to the head, while a clear area containing the nebenkern is found between this and the tail. The tail is very delicate and is seen only in material that has been counterstained. The details of spermiogenesis could not be followed accurately enough to permit further description or presentation of figures.

There is no indication in my material that indifferent cells of the gonad differentiate into sex cells, as described by Witschi ('21) and Swingle ('21) for frogs and by Hargitt ('24 and '25) for the salamander and rat. The germ cells in Sphaerium seem to be continuous from their original seg-

Fig. 49 Diagram of an embryo in the hatching stage, to show the relation of most of the structures in the body, made from a whole mount of an embryo dissected from the gills. × 50.

Fig. 50 Oblique section through a twelve-cell stage in which one micromere shows a quadripolar spindle. × 35.

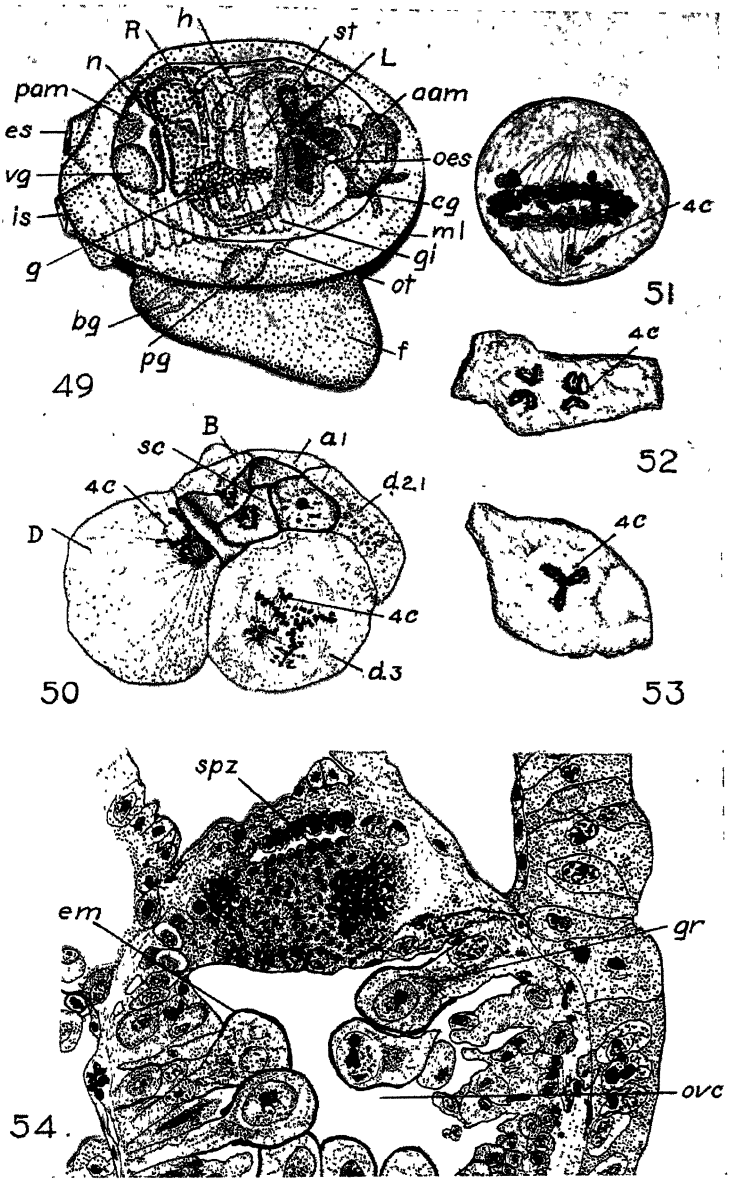
Fig. 51 Secondary spermatocyte in early anaphase, showing four masses of chromatin (*4C*) which precede the remainder in passage to either pole. × 1200.

Fig. 52 A multipolar cell in the nephridium, showing the four chromatin masses that precede the remainder in passage to either pole. × 600.

Fig. 53 A tripolar spindle in a brood-pouch cell, showing same chromatin behavior as figures 51 and 52. × 600.

Fig. 54 A portion of the gonad of a recently matured specimen, showing close relation of male and female regions of the gonad. × 275.

[1] Sphaerium striatinum from the vicinity of Columbia, Missouri, produce spermatozoa with heads approximately 50 per cent longer than sperm of specimens collected at Woods Hole, Massachusetts, although these were identified as belonging to the same species (compare p. 546).



Figures 49 to 54

regation during cleavage to the time of maturation. I have found no evidence of primordial germ cells giving rise to anything except functional germ cells. It is possible that some primordial germ cells are lost as a result of the method by which nutritive cells become incorporated into the cytoplasm of the oocytes. Such cases are described by Stauffacher ('93), although I have not observed them in my material. Whole cells adjacent to a growing oocyte do become incorporated into the oocyte cytoplasm, but so far as I can determine, these nutritive cells are epithelial cells and not primordial germ cells. As previously described, these two types differ greatly in structure. There is no indication of a precocious maturation of the germ cells in Sphaerium as described by Witschi ('21) and others. Once maturation begins, it is apparently a continuous process during the life of the individual, though the rate may vary seasonally. Spermatozoa produced by very young animals are in no way different from those in older specimens except that the total number is smaller because the gonad is smaller. The same is true of the ova. No difference can be seen in ova of young and full-grown specimens of Sphaerium. Maturation begins only after the young have been set free from the gills and have attained a length of about 2.5 mm. Gilmore ('17) states that in Sphaerium simile young are not produced until the animals reach a length of 10 mm., though the gonads are fully formed in individuals 6, 7, and 8 mm. in length. This differs from Sphaerium striatinum, in which young are present in the brood pouches of 3-mm. specimens and are set free from specimens of 4 and 5 mm. in length.

No clues have been obtained suggesting the factors that determine which cells of the gonad will become spermatozoa and which will become ova. The cytological methods used reveal no differences in the primorial germ cells that forecast later differentiation into either ova or spermatozoa. The work of McClung ('02), Montgomery ('04), Stevens ('08), Harvey ('16), Carothers ('17 and '21), and many others has demonstrated the correlation between sex and specific chro-

mosomes. I have found no description of such differences for monoecious animals. Schrader ('21 and '28), Bridges ('21, '22, and '25), and others show that environmental factors are in part responsible for sex or that it is a matter of the relative quantitative action of sex-determining factors not definitely located in a single pair of homologous chromosomes. The relations of sex to chromosomes in Mollusca seem to have been investigated only in some Gastropoda (compare Schrader, '28, pp. 67–71).

Discussion

Primary cellular differentiation or segregation of the primordial germ cells has been described in representatives from many groups of animals. Evidence has been presented to show that this is the general occurrence, although exceptions can be found. In forms that show an early segregation of primordial germ cells, whether these cells later give rise to definitive germ cells or not, there follows a period of inactivity. Hegner ('14, p. 376) characterizes this period of inactivity as one of separation of the germ cells into two groups, cessation of cell division in the primordial germ cells, and active or passive changes in position before the formation of definitive sex cells. This period of inactivity occurs during the growth of the embryo and continues until the larval stage is almost attained.

In claiming that early segregation of germinal material occurs in Sphaerium, it is not my intention to imply any activity on the part of this material during segregation or any continuity of unchanged egg substance that is responsible for the production of germ cells. It is my belief that cleavage and mesoderm formation in Sphaerium result in the separation of other potentialities of development from that of producing germ cells. There is no evidence of activity on the part of the primordial germ cells until they have reached their place of development in the gonad.

According to Hargitt ('25), the production of germ cells in vertebrates is a matter of cellular differentiation compar-

able with that of somatic cells, and not one of early ontogenetic segregation. The case in Sphaerium agrees with such an interpretation, except in one respect: while other types of cells differentiate from embryonic cells early in ontogeny, the germ cells are inactive and retain their embryonic condition until they differentiate into functional germ cells in the adult. From this viewpoint functional germ cells are not to be considered as the first cells in which differentiation occurs in the developing organism, but as the last cells to reach a functional state.

In Sphaerium there is no evidence that somatic cells ever give rise to germ cells. If a cell is specialized for the performance of some bodily function, it seems incapable, in Sphaerium at least, of giving rise to a new individual. Germ cells are the only cells having this capacity. The end result of meiosis is comparable to differentiation of somatic cells in that functional cells are produced. In Sphaerium, so far as can be determined, all functional germ cells are the lineal descendants of primordial cells that have been 'left behind,' as it were, while cellular differentiation was occurring in other parts of the body. That these primordial germ cells have lost all their capacities for differentiation, except that for the production of gametes, is shown by the fact that they seem never to give rise to any type of cell except gametes.

In cases where so-called dedifferentiation of somatic cells is described (Gatenby, '16; Kingery, '17; Spehl and Polus, '12, etc.), it is possible to interpret these changes in terms of cells that have retained capacities for differentiation in more than one direction. Under a given set of developmental conditions one of these potentialities has been expressed in beginning differentiation toward a particular type of cell, but only to a limited extent. If differentiation has not been completed, changing the environmental conditions of the cell may make possible the expression of an entirely different capacity for differentiation. The cell may complete differentiation in this new direction and lose the characteristics it had acquired temporarily. The evidence is inadequate to

show that a highly specialized cell, like a muscle or a nerve cell, having a limited function can become dedifferentiated and that the resultant undifferentiated cell can redifferentiate into an entirely different type of cell with different function, although such cases have been described (compare Vandel, '22, p. 428). Until individual cells have been followed through the process of dedifferentiation and redifferentiation such evidence will not be conclusive. The changes described as dedifferentiation may as well be interpreted as the degenerative changes preceding death. In other cases, like that of the planarians just cited, it is difficult to rule out the possibility that 'formative cells' (compare Curtis, '02 and '28) have not replaced those that seem to have arisen by dedifferentiation. The great body of evidence indicates that complete cell specialization is irreversible. It is true, however, that cells do retain more than one potentiality for complete specialization if they are not too highly specialized. Germinal epithelium cells belong to this group in the vertebrates. They may become peritoneal cells, germ cells, Sertoli cells, etc., as shown by many workers. In Sphaerium the only source of germ cells is the primordial germ cells.

Production of functional germ cells is, then, a matter of cell differentiation and specialization, comparable in a sense with tissue differentiation in other types of cells, but differing in that it is delayed. The so-called early segregation of germ cells is merely suspended activity and delayed differentiation. Although we know little of the causes of differentiation, we do know the time of differentiation in many cases. The other tissues in Sphaerium differentiate, so that the undifferentiated line of primordial germ cells is conspicuous early in ontogeny. That no continuous line of germ cells is evident in some vertebrates is not to be denied. The question is whether the germinal material is not only lost to view, but is lost entirely and appears de novo from some specialized body cell. It may be true that the potentiality for producing germ cells exists, perhaps, in several kinds of cells, and that the particular set of conditions necessary for the expression

of this potentiality occurs only in a limited portion of the body, the gonads, and only at certain cyclic periods in the life of the organism. Gatenby ('23) thinks this is true for Amphibia. Germinal continuity of some sort seems to exist, as evidenced by the fact that at certain periods cells become differentiated for reproduction. Whether cells carrying such potentialities for specialization can be recognized early or late in ontogeny seems of little consequence. Their behavior in the process of maturation and fertilization and in subsequent development into a new individual is strikingly similar in their fundamental events in all multicellular animals.

The claim that egg cytoplasm is passed unchanged through a continuous line of cells that will differentiate into germ cells cannot be substantiated. Development is a continuous process of change. Cleavage separates the cytoplasm of the egg into portions whose potentialities for normal development are different. Every change that occurs in development affects the materials in the cells. The passage of the cytoplasm of a particular cell, unchanged, to the descendants of that cell is not in keeping with our concept of protoplasm existing in a state of dynamic equilibrium.

The zygote receives from each parent certain capacities for development. As development proceeds new capacities are acquired. In addition to these potentialities, the environmental factors determine the nature and extent of expression of the capacities inherited in the zygote and acquired in development. In view of these facts, it is almost inconceivable that germinal material remains constant in substance or behavior throughout its history in one generation. Egg cytoplasm possesses certain capacities for interaction with the nucleus that result in cellular differentiation. One of these is the capacity to produce germ cells under normal conditions at a definite time and place in the organism. The time varies greatly, just as the time of differentiation of other types of cells varies in different animals. That a particular region can be recognized as the part from which germ cells ultimately will develop does not imply that this cytoplasm re-

mains unchanged throughout subsequent cellular generations. In Sphaerium the inherent capacity for differentiation into germ cells descends through a particular line of cells to the primordial germ cells.

In many cases germinal continuity has been claimed, but is incompletely demonstrated. In other cases discontinuity has been claimed. The question of germinal continuity, therefore, remains unsettled. Direct cellular continuity can be demonstrated in some animals, Ascaris, Miastor, and other insects, Sphaerium, etc., but is believed not to exist in others like certain Amphibia, Mammalia, etc. If germinal continuity exists in all animals, it must be similar to the sort of continuity postulated for heritable somatic characteristics. The argument from facts to conclusion supporting the theory of germinal continuity is of the same nature as that supporting the gene theory in heredity and the atomic theory in chemistry. Both the gene theory and the atomic theory have undergone as much modification at the hands of investigators since their first promulgation as the original theory of germinal continuity of Weismann. It cannot be maintained in all cases that primordial germ cells are recognizably different from other embryonic cells or that they are present before gonad formation. The theory that 'germinal continuity' exists because something in the life cycle of animals behaves as if it were continuous is open to criticism. The gene theory, 'crossing-over,' and the atomic theory can be criticized for the same reason. I shall not discuss the vitalistic aspects of these theories. Considered as working hypotheses, the value of these theories has been demonstrated by the history of their biological accomplishment. Such hypotheses have undergone much modification in the past and no doubt will be further modified as new facts become known.

SUMMARY AND CONCLUSIONS

The history of the germ cells in Sphaerium is traceable from the fertilized egg to sexual maturity in the new individual, making a complete cycle.

By cell lineage it can be shown that the germ cells are derived from the cell *D*, of the four-cell stage, and are definitely set aside as primordial germ cells by the third division of the paired mesoderm cells, *M* and *M.1*, arising from the single cell *M*, which in turn is the fourth micromere produced by the cell *D*.

After the formation of the mesoderm, the subsequent history of these primordial germ cells can be determined with no great difficulty, since they differ in size and appearance from other cells of the embryo.

Following a single division of the two original right and left germ cells, the two primordial germ cells on each side of the embryo remain inactive until after the differentiation of the nephridia and the beginning of differentiation of the pericardial cavities. During this period their position is changed by the development of other structures.

At the end of the inactive period mitotic division is resumed by the primordial germ cells, and this marks the beginning of gonad formation. Observations on gonad formation in this genus confirm those of earlier investigators.

Maturation is first evident in the female portion of the gonad. Cytoplasmic growth occurs and there is meager evidence of meiotic changes in the nuclei of cells in this region. Maturation begins in the male portion before full-grown oocytes are present in the ovary.

There is no indication of alternating activity in the male and female regions of the gonad in this species, since mature individuals always show male and female sex cells in all stages of development throughout the year.

The primordial germ cells, which are visibly different from somatic cells early in development, do not lose their identity at any later period. Only two kinds of cells are present in the gonad: primordial germ cells and peritoneal cells. New germ cells arise only from pre-existing germ cells and primordial germ cells apparently do not transform into somatic cells in the gonad.

The germ-cell cycle in Sphaerium may be divided into five periods with well-defined limits:

1. The original appearance, with the formation of two and then four primordial germ cells during the cleavage stages.

2. An inactive period, during which the two primordial germ cells on either side of the embryo do not divide, but are carried to the place of gonad formation by growth in other regions.

3. A multiplication period in the developing gonads continuing throughout the life of the animal and producing an indefinite number of primordial germ cells.

4. Maturation, including oogonial and spermatogonial growth, and the meiotic nuclear changes.

5. Fertilization.

The details of maturation and the chromosomal number could not be determined, because the chromosomes are small and clumped together.

No indication could be found of chromosomal differences in the primordial germ cells that forecast which cells would become ova and which spermatozoa. No theories are offered to explain this differentiation.

Although a definite cellular continuity for the capacity to produce functional germ cells seems demonstrable in Sphaerium, this does not imply that egg cytoplasm passes unchanged through the cleavage stages to the primordial germ cells.

It is believed that the characteristics by which the primordial germ cells are recognizable early in cleavage arise because these cells retain the capacity for further differentiation, in contrast with somatic cells which lose this capacity as they differentiate. Thus the production of functional germ cells is thought to be a process of differentiation comparable with that which occurs in other cells at a much earlier stage of development. In other respects the germ-cell differentiation is comparable to differentiation of somatic cells.

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NOTES ON THE POSTNUCLEAR, ACROSOME-SEAT GRANULES, AND 'VACUOME' IN DESMOGNATHUS FUSCA SPERMATOGENESIS

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FIFTEEN FIGURES

AUTHOR'S ABSTRACT

The acrosome in Desmognathus, Spelerpes, Plethodon, salamander, Amphiuma, etc., is attached to the nucleus in connection with an acrosome seat, which forms a shallow cup, traced back to a number of granules in the early spermatid. A postnuclear plate is present in the above-mentioned urodeles, and is derived from a small number of minute granules which assemble in the spermatid and become fixed onto the nuclear membrane. The centrosomes of the spermatid are visible intravitam. The 'vacuome' is formed of minute neutral-red-staining globules embedded in the idiozome. No connection appears to exist between mitochondria and Golgi bodies, as is postulated by the vacuome-chondriome hypothesis (Parat).

INTRODUCTION

There are a number of points in the spermatogenesis of Urodela, as already recorded, which call for reexamination: *a*) The absence of mitochondria from the 'middle-piece.' *b*) The claim by McGregor that the sphere takes part in the formation of the 'middle-piece.' *c*) The nature and function of the ring of granules around the acroblast, which were noted by Bowen. *d*) The question of the postnuclear body, already raised elsewhere. *e*) The fate of the dictyosomes of the Golgi apparatus. *f*) The nature of the 'vacuome' in Urodela. *g*) The question as to whether in these very large cells, any relationship between Golgi cortex and mitochondria can be noted, as is necessary for Parat's vacuome-chondriome hypothesis. *h*) The ability or inability to see the centrosomes intravitam in such large cells (Fry). *i*) Whether the central bodies of spermatocyte mitosis and the central bodies of the developing spermatid are one and the same thing (Fry).

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The writer wishes to acknowledge the assistance received from preparations generously lent by Dr. W. R. Coe. Dr. A. Petrunkevitch and Dr. V. Twitty also kindly assisted in identifying the species used.

LITERATURE

The outstanding modern study of urodele spermatogenesis is that of Tullio Terni, who worked out both Golgi elements (his formazioni periidiozomiche) and mitochondria perfectly, except for the later stages of spermateleosis, during which his account of the Golgi elements is incomplete. Terni also failed to find the postnuclear bodies and the acrosome-seat granules.

F. Meves, thirty-three years ago, gave a very clear and excellent account of the behavior of the centrosomes. He depicts what the present writer considers to be the postnuclear plate (fig. 19), and the acrosome seat (figs. 24 to 27) at a late stage.

Over thirty years ago, McGregor described the spermatogenesis of Amphiuma, and as will be seen, he found and described the postnuclear granules and plate, and figures the acrosome seat at a late stage.

Bowen, in 1922, used the modern silver and osmic methods on a limited amount of material of Plethodon cinereus, and showed that the idiozome (Golgi elements) does not disappear at the time supposed by McGregor. Bowen also saw the acrosome-seat granules, but did not realize their significance. He failed to find the postnuclear plate discovered by McGregor in Amphiuma. The work of these four cytologists will be further mentioned in the text of this paper.

Recently, Jan Hirschler has used the supravital neutral-red method and shown some small red granules within the idiozome. These correspond to Parat's 'vacuome,' and cannot be found except by neutral-red staining.

MATERIAL AND METHODS

The material used consisted of testes of Desmognathus fusca, Plethodon cinereus, and Spelerpes ruber, fixed toward the end of September. This is apparently a little late, the best time in the eastern states of America being probably at the end of August. Quite enough stages were got in the material fixed in September, but such testes contained mainly spermatocytes and ripe spermatozoa.

Three chief methods were used: Kolatschew, counterstained in neutral red, Champy iron-alum haematoxylin, and Da Fano's method. A good many supravitally stained preparations in neutral red were used. These showed some small red globules inside the idiozome, but could not be considered of any special help or value.

Many smears of ripe spermatozoa, both living and fixed, were made.

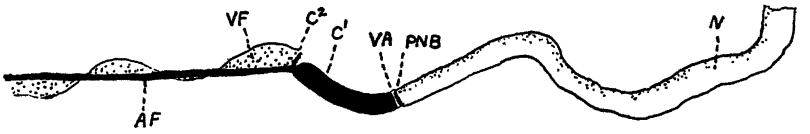


Fig. 1 Ripe spermatozoon of Desmognathus fusca, showing parts, nucleus (*N*), postnuclear plate (*PNB*), clear area (*VA*), head centrosome (C^1), upper part of second centrosome (C^2), axial filament (*AF*), vibratile filament (*VF*).

THE RIPE SPERMATOZOON

Special study was made of the ripe spermatozoa of Desmognathus fusca, though those of Plethodon and Spelerpes were carefully examined, as well. In figure 1 the middle region of a Desmognathus sperm is drawn; the nucleus is at *N* and passes down to a stainable end structure at *PNB*, which is the postnuclear body; next to the postnuclear body is a non-staining space (*VA*), quite clear in the living sperm, but usually impossible to find in sperm smears fixed in Bouin and stained in iron-alum haematoxylin. It is possible to see this space in good chrome-osmium sections or in smears fixed in osmic vapor, but it does not stain with neutral red; behind this space is the large refringent head centrosome (C^1),

which is easily seen in the living spermatozoon. Next comes the axial filament (AF), to which is attached the vibratile filament so-called, which appears to begin from an outward process which has been identified as the top of centrosome 2 (C^2) derived from the ring centrosome. If and when the spermatozoon breaks in half during the operation of smearing, the line of separation is practically always at VA, where the space is, the attachment at C^2 apparently being stronger.

INTERRELATIONSHIP BETWEEN GOLGI BODIES AND MITOCHONDRIA

These urodele cells are very large, and in the easily made Kolatschew preparations the Golgi apparatus and the mitochondria can be studied without difficulty from spermatogonia to ripening spermatid. Here is material with which the idea of Parat, that the dictyosomes are modified mitochondria, can be tested as in no other order.

In Desmognathus the Golgi apparatus can be traced from primary spermatogonium to spermatid, clearly without any visible connection with the mitochondria. The latter either do not stain at all, or stain differently; they are elongate, perfectly characteristic in shape, and bear no resemblance to the chromophil part of the dictyosome. The suggestion of Parat that the dictyosome is a modified mitochondrion receives no support from a study of the spermatogenesis of this salamander. If mitochondria metamorphose into Golgi elements, this must take place at some other epoch in the germ-cell cycle.

THE POSTNUCLEAR BODY

McGregor, in his study of Amphiuma, describes what the present writer considers to be the postnuclear body, under the name 'globule' or 'globular body.' McGregor's description is quite straightforward up to the disappearance of the sphere substance, and the present writer is almost entirely in agreement with him. As we shall see, the sphere does disappear as such, not exactly in the manner McGregor claimed, but in a different way, not by absorption into the 'middle-piece' (at least entirely), but by dispersal in such a manner

that the individual parts no longer constitute a single stainable area as before.

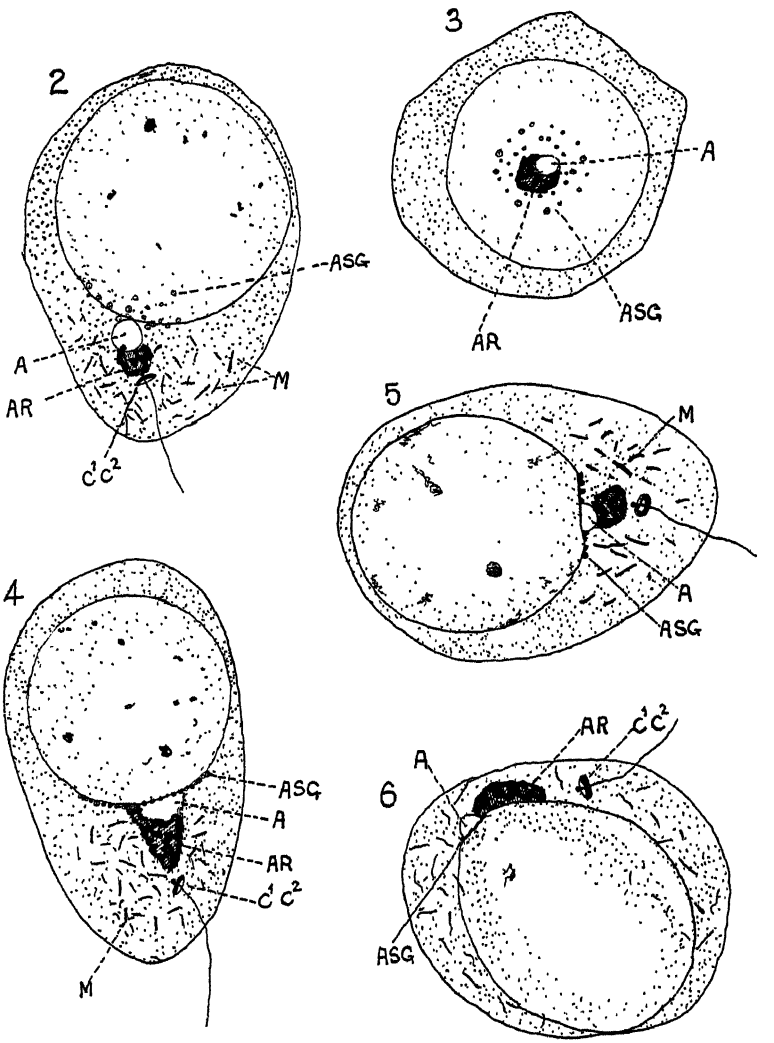
The postnuclear body in Desmognathus is not easy to follow. Once formed, and while the spermatid nucleus is still broad and short, the postnuclear body is quite clear. Its presence in the ripe spermatozoon is also easily detected, but the anlagen in the spermatid could not be found until fixed onto the end of the spermatid.

Writing of his 'globular body' (the writer's postnuclear body), McGregor says, "At first it is thin and flat, and may present the form of two or three minute flattened masses which fuse later."

He writes also, "The next step is the passage of the globular body into the nucleus to form the middle-piece, and at the same time the basal knob (deeper centrosome, C^1) becomes embedded within the globular body."

Now in modern Champy iron-alum haematoxylin preparations, little of this story in Desmognathus can be worked out, but in the older Flemming-acetic and long-treatment iron-alum haematoxylin slides, the elucidation of this part of spermatozoon formation becomes feasible.

In figure 7 is a free-hand drawing of the end of an elongating spermatid, showing a row of granules (*PNG*) attached to the nuclear membrane. This figure corresponds closely to what McGregor has drawn in his figure 26, and these granules are regarded by the present writer as exactly homologous with similar bodies already noted in other orders (Gatenby, '29). It has been impossible to identify these granules at an earlier stage. This might have been done in the Da Fano preparations, had they been better. At a later stage, after coalescence of the granules, as shown in figure 8 (*PNB*) the structure becomes very clear and there can be no question as to its presence. In some examples the head centrosome appears to become embedded in the postnuclear plate or body, but later, on further growth, it is demonstrable that the two structures are quite separate. Of this period Bowen writes:



Figs. 2 to 6 Figures 2 and 3, early spermatid showing deposition of acrosome (*A*) by Golgi-idiozome complex (*AR*), assembly of acrosome-seat granules (*ASG*), and growing centrosome parts (c^1 and c^2), mitochondria (*M*). Figures 4, 5, and 6, aggregation and union of acrosome-seat granules to form acrosome seat (*ASG* in fig. 6), deposition of acrosome, and beginning of flowing down of Golgi apparatus (*AR* in fig. 6).

I have been unable to find any evidence of an actual penetration of the nuclear wall by the 'middle-piece' as described by both Meves and McGregor. The centriole seems rather to be merely in contact with the nucleus, as is the case in many other animal sperms.

Bowen has failed to find the postnuclear body, which in Plethodon, as in other forms, does pass into the outline of the nucleus as described by Meves and by McGregor. The latter's account as mentioned above is entirely in agreement on this point with what the present writer has found. The centriole, as Bowen claims, does not pass into the nucleus, but may in some cases become temporarily embedded in the postnuclear plate, when the latter is first formed. It is this which was mistaken for Meves as the passage of the 'middle-piece' into the nucleus.

Finally, the postnuclear plate becomes the thin, but very chromophil line, shown in figures 1, 9, 11, 12.

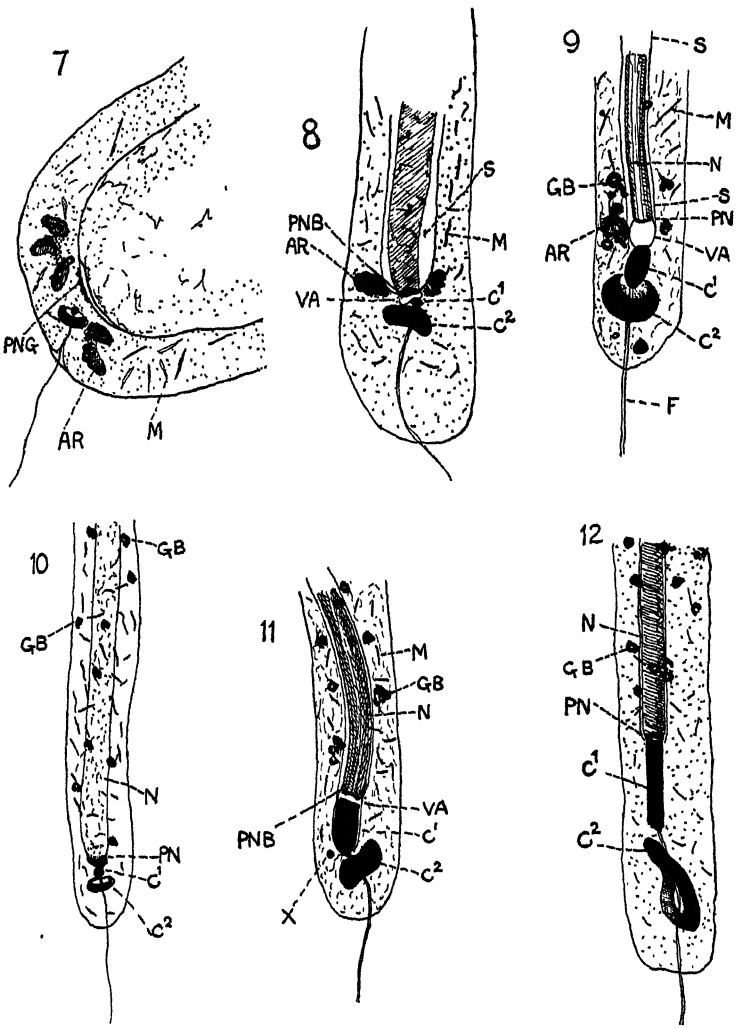
THE GOLGI APPARATUS-IDIOZOME COMPLEX AND THE 'MIDDLE-PIECE' REGION

One of the objects of undertaking this work was to see, if possible, whether McGregor's account of the origin of the 'middle-piece' was correct. McGregor writes, "I find the middle-piece in Amphiuma to be chiefly derived from the remnant of the sphere, though the deeper centrosome (end-knob) does become embedded in it." Later on, McGregor writes:

> It is impossible to state with absolute certainty from which it (McGregor's 'globular body' or the present writer's postnuclear plate) is derived, though the most probable explanation seems to be that it is formed from the sphere substance for the latter disappears as the globule enlarges.

McGregor's account, according to the present writer, is correct so far as the facts are concerned, but the conclusion drawn is incorrect.

In Kolatschew preparations it is possible to show that, as McGregor claimed, the Golgi apparatus (idiozome) approaches the 'middle-piece' region and seems to wrap around it, even more closely than is depicted in figures 7 and 8. At this time, matters are complicated by the appearance of



Figs. 7 to 12 Further development of spermatid, showing earliest stage in assembly of postnuclear granules (*PNG*) in figure 7, and grouping of Golgi bodies around 'middle-piece' region. Figures 8, 9, and 11, appearance of vesicular area (*VA*), and rapid growth of centrosomes. Coalescence of postnuclear bodies in figures 8, 10 (*PNB*, *PN*) to form thin postnuclear plate of later spermatid (figs. 9, 11, and 12). Upward migration of Golgi elements with elongation of spermatid (figs. 10, 11, and 12). Beginning of twisting and lengthening of ring centrosome (C^2).

another structure, namely, a vesicle marked *VA*, in figures 8 and 9; the exact source of this vesicle could not be elucidated, and in inferior preparations it could not even be found (e.g., fig. 10), but it is present in the adult spermatozoon as shown in figure 1. McGregor draws what is the same space in his figures 26 to 31, and writes, "a clear space filled with Karyolymph soon appears under the (nuclear) membrane, at the posterior end of the nucleus." This feature of the spermatid at this period has puzzled the present writer considerably. In Doctor Coe's preparations of Desmognathus, in the stage drawn in figure 9, there is a large vesicle (*VA*) here, which occurs constantly in all spermatids in a bundle. In some examples it is larger than in figure 9, and undoubtedly grows considerably between the stages depicted in figures 8 and 9. Thereafter, it begins to shrink, until it is a narrow space between the postnuclear plate and the head centrosome, as shown in figures 1, 11, and 12.

Now between the stages drawn in figures 7 and 8, a space (*S*) appears around the entire spermatid nucleus, excepting at the extreme head end, where the acrosome lies, and the middle region, where the bodies already mentioned are formed. This space has been overlooked by some authors, but it is undoubtedly present. The difficulty then arises as to what connection this space has with the previously mentioned region (*VA*) shown in figures 8 and 9, etc.

There is the temptation similar to that into which McGregor fell, to assume, because the Golgi-idiozome complex approaches the 'middle-piece' region just when the space (*VA*) appears, that the latter is produced by the idiozome. The writer has found it impossible to disprove McGregor's claim that the 'middle-piece' gets part of its substance from the idiozome. It is easy enough with modern preparations to show that the Golgi bodies move away from the 'middle-piece' region and are not absorbed as was thought, but to deny that they leave something behind would be unwise. For one thing, what are they doing at all in that region? On the analogy of other forms, such as molluscs, mammals, annelids, etc., the

postnuclear bodies are not produced by the idiozome; the point is, however, whether this space contains a fluid contributed by the idiozome. If so, McGregor's main contention would be right. At present the writer cannot offer any better exposition of the matter.

On the question of the fate of the Golgi elements after breaking up and dispersal up the lengthening spermatozoon (fig. 10), there is no doubt that by current smearing methods it is impossible to find the dictyosomes in the adult spermatozoon. As will be mentioned later, Terni, by special treatment, has shown mitochondria to be present in the adult spermatozoon. In view of this, it would be unwise to claim that Golgi bodies are absent from the adult sperm, especially since, first, the writer could not be sure that all the Golgi remnants went into the scaled-off protoplasm of many of the spermatozoa, and secondly, since the dictyosomes occupy much the same position during spermateleosis as the mitochondria, which we know from Terni, actually do persist in the adult spermatozoon. It is obviously a matter which might be settled by examination of the early stages in fertilization.

ACROSOME SEAT

The most interesting part of this study has been the working out of the fate of a number of granules which were seen by Bowen, and which must be present in Amphiuma, as is shown by McGregor's figures 27 to 32, in Geotriton fusca (Terni, fig. 48 a), and in the European salamander (Meves, figs. 24 and 25). McGregor, Meves, and Terni seem to have failed to note this structure when in the remarkable granular form.

In figures 2, 3, and 4 of the present paper, a circle of granules (*ASG*) is very noticeable around the forming acrosome. They are drawn in Bowen's figures 4 and 6; of them he writes:

> I have also found in the spermatids a collection of granules which seem to be preserved best in the absence of acetic acid. During the formation of the acrosome these granules are arranged in a circle around the acroblast. . . . This arrangement is a constant and striking one. The nature and fate of these granules is not known.

At first they were mistaken by the present writer for the postnuclear granules, but closer attention to them showed that, as in figures 5 and 6, they eventually close up together and form an acrosome seat, which can be followed for a long time in the formation of the ripening spermatozoon.

Similar acrosome seats have not been described in any vertebrates, so far as the present author is aware, but there are parallel structures in certain Mollusca. Probably in most animals the development of acrosome seats is screened by the acroblasts, which in forms other than the Amphibia are bigger in proportion to the nucleus. The granules, as Bowen mentions, are best seen after acetic-free fixatives, but in the case of chrome-osmium mixtures, a very thorough washing out of the material in running water is necessary to get a good stain with alum haematoxylin. These granules show well in Doctor Coe's Flemming-acetic-fixed preparations.

The function of the acrosome seat in salamanders appears to be the cementing of the acrosome onto the nucleus.

THE ABSENCE OF MITOCHONDRIA FROM THE 'MIDDLE-PIECE'

Whatever various authors may write with reference to the origin of the 'middle-piece,' there is a consensus of opinion that the mitochondria do not enter into the formation of the 'middle-piece.' This is very curious, and does not appear always to have been remembered by those who have discussed the function of the mitochondria in other spermatozoa. Tullio Terni, in Geotriton fusca, gives what may be considered the clearest account of the mitochondria. He shows[2] that though mitochondria may seem to be stripped off, or absent from the adult spermatozoon, they can be shown to be present by treating the sperms in 7 per cent NaCl for three days, and then fixing in chrome-osmium and staining in Altman's fuschsin. The mitochondria occupy a region reaching from the top of the head centrosome to well up the nucleus. Now in Bouin or Champy smears of Desmognathus sperms, no mitochondria

[2] The present writer has failed to confirm Terni's claim. Several strengths of salt solution were used, as well as distilled water.

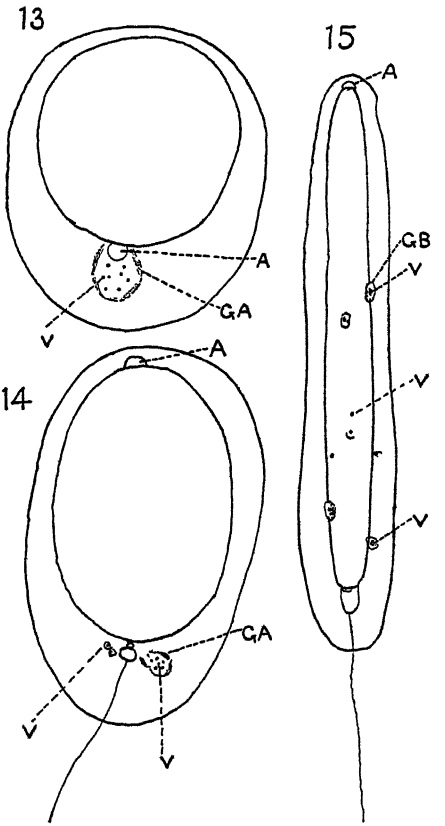
can be seen on the adult spermatozoon, but in many but not all Spelerpes spermatozoa a globule of protoplasm does exist on some part of the nucleus, but more commonly around the middle region. In Rana nigromaculata Jun-ichi Morita has shown that mitochondria exist in the adult spermatozoon in the granular form, dotted over both nucleus and tail, but do not repose directly upon a true middle-piece as in the sperms of so many other organisms.

THE VACUOLAR SYSTEM

A number of very fine red granules can be stained within the idiozome by means of neutral red (fig. 13). These correspond in every way except in size to the neutral-red granules depicted for Triton cristatus by Jan Hirschler. It was not possible in the material used either to stain the acrosome red or to show that the middle-piece vesicle was derived from the vacuoles. In some cases examined, the neutral-red granules had separated from the idiozome of the spermatid, and lay toward the front half of the elongating nucleus. In Desmognathus the acrosome does not appear, in the fresh condition, to contain a central more solid bead, as described for Triton by Hirschler or for Amphiuma by McGregor. It is, rather, a limpid vesicular structure of an extremely delicate nature. In Bowen's plate 1, figure 11, the acrosome of Plethodon is also drawn as a vesicle without a central granule. This probably constitutes an important difference between the acrosomes of Desmognathus and Plethodon on the one hand and Amphiuma and Triton on the other, from the point of view of stainability at this period in neutral red or haematoxylin, both of which are taken up by the central granule of the acrosome. Terni, in Geotriton fuscus, does not figure a central granule in earlier stages of acrosome formation.

In spermateleosis the neutral-red vacuoles pass down with the idiozome (Golgi remnant) to the 'middle-piece' region (fig. 14), and with the dispersal of the dictyosomes can still be seen, usually associated with the individual pieces of the Golgi apparatus, but sometimes separate (fig. 15).

In the ripe sperm no structures particularly active in taking up neutral red could be discovered, and the conclusion seems forced upon us that the vacuolar system does not persist as such in the adult spermatozoon. But in view of Terni's demonstration of mitochondria in the adult spermatozoon, it seems advisable to await for the results of newer techniques before endeavoring to express a final opinion.



Figs. 13 to 15 'Vacuome' of Desmobranchus, stained supravitally in neutral red. Figure 13, spermatid showing formation of acrosome (*A*), with eight minute neutral-red globules (*V*) inside Golgi-idiozome complex (*GA*). Figure 14, beginning of break-up of Golgi apparatus. 'Vacuome' within the fragments. Figure 15, 'vacuome' with and without accompanying Golgi elements (*GB*) in older spermatid.

THE CENTROSOMES VISIBLE INTRAVITAM

The ring and the head bead centrosome of Plethodon, Spelerpes, and Desmognathus can be seen very clearly in living cells, as they are so large and refringent. In the same manner the centrosomes of moths (Abraxas) and molluscs (Helix and Arion) may be seen—if less easily, still indubitably—in the lengthening spermatids.

In his latest paper on the centrosome, H. J. Fry writes:

> These most recent results indicate that in fertilized Echinarachnius eggs, which are probably typical of echinoderms generally, the so-called central bodies, which are assumed to be the formative foci of the mitotic figure and its most persistent element, actually have no existence in the living egg. . . .
>
> The extent to which the (above) conclusion applies to central bodies in other animals remains to be seen, but since the new results have been established in cells heretofore assumed to have typical central bodies, at least some of the usually accepted facts have been called into doubt. The question immediately arises: what is the significance of the newer result with regard to central bodies of spermatocytes? In answer to this, the suggestion is made that central bodies of spermatocytes may have less to do with mitotic figures than is generally assumed; they are primarily blepharoplasts of the sperm's axial filament, perhaps playing no active rôle in mitosis.

While the writer has not yet carried out any special investigation with echinoderm material, on the analogy of other forms Fry's suggestion that the centrosome is an artefact cannot be taken seriously.

In the matter of the centrosome of spermatocytes in general, Fry's idea that the central bodies may have less to do with mitotic figures than is generally assumed is not supported by any evidence. Quite the contrary is the case, the central bodies being traced easily in many forms such as Desmognathus from spermatocyte spindle to the head of the outgrowing flagellum. In the classic case of the lepidopterous spermatocytes, the flagella grow out before the maturation division from central bodies, which later function as the center of the mitotic asters. No case is known to the writer in which the central bodies of spermatocytes wait aside, after-

ward providing the source of the outgrowing flagellum (while some other 'amphiastral artefacts' preside over maturation mitosis).

SUMMARY

1. The acrosome in Desmognathus, Spelerpes, Plethodon, salamander, Amphiuma, etc., is attached to the nucleus in connection with an acrosome seat, which forms a shallow cup in which the acrosome reposes.

2. The acrosome seat is formed from a number of separate granules which run together, fusing to form a solid body.

3. A postnuclear plate is present in the ripe spermatozoa of Desmognathus, Plethodon, Spelerpes, Amphiuma, etc. It was correctly described by McGregor under the name 'globular body.' The postnuclear plate is not derived from the Golgi-idiozome complex.

4. The postnuclear plate is formed by a small number of minute granules which become attached to the end of the spermatid nucleus after the centrosomes have drifted around to that region.

5. The centrosomes of the Desmognathus, etc., spermatid are visible intravitam, without staining.

6. There is a 'vacuome,' in the form of minute neutral-red-staining vacuoles lying inside the idiozome. With the dispersal of the Golgi bodies, the vacuoles usually adhere to the chromophobe parts of the former.

7. The Golgi apparatus-idiozome complex does not become incorporated into the middle-piece, though it was not possible to say whether any part of the middle-piece was derived from some secretion from the idiozome.

8. No visible relationship appears to exist between Golgi dictyosomes and mitochondria at any time in the spermatogenetic cycle.

9. The dictyosomes of the Golgi apparatus go black in formalin-silver nitrate, and these, and not the 'vacuome,' constitute the true Golgi apparatus.

10. No mitochondria directly enter into the formation of the middle-piece.

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INSECT DEVELOPMENT

II. MITOTIC ACTIVITY IN THE GRASSHOPPER EMBRYO

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TWO FIGURES

AUTHOR'S ABSTRACT

The onset of hibernation in Melanoplus differentialis eggs kept at high temperatures is accompanied by a fairly abrupt cessation of cell division. The total number of mitotic figures present in the different individuals of a series of embryos of this grasshopper, from eggs kept for eighteen to twenty days at 25°C. from the time of laying, was found to vary between 1000 and 4400. In contrast to the conditions found in eggs of this age, embryos from eggs kept twenty-seven days at 25°C., as well as those older and still in the hibernating state, contained few or no dividing cells. During the course of the work it was found that, in rare cases, hibernation failed to occur, a few or many of the eggs developed without a pause when kept at 25°C., and hatched at approximately the thirty-eighth day. The detection of variations in the rate of mitotic activity over short periods, as originally planned, was found impossible, because of the comparatively small number of embryos used.

INTRODUCTION

The following study was made in an effort to see whether definite rhythms in the rate of mitotic activity could be detected, over short periods, in the developing embryo of the grasshopper, Melanoplus differentialis. Although such rhythms were not found, it may be worth while to record the results and to show the extent of the variation encountered in this material.

Near Philadelphia Melanoplus differentialis lays its eggs in the late summer and fall. These remain in the ground over winter and hatch the following spring. If such eggs, immediately after being laid, are placed at a moderately high temperature—say, 25°C.—and kept there, they will develop for about three weeks. Growth then stops rather abruptly and the egg remains in this quiescent condition for several months. Examination of the embryo at this time shows it to extend a little less than half the length of the egg, with the head, appendages, and ventral portions of the thorax and abdomen well developed, but with the dorsal surface still

incomplete and open to the yolk. The head lies close to the caudal pole of the egg and the embryo is, to all appearances, ready to undergo blastokinesis; that is, to revolve around the caudal end of the egg and to head up toward the cephalic pole. This, however, does not occur. Hatching takes place only after a prolonged period of dormancy and the time at which it occurs varies considerably even with the eggs in a single pod. In rare instances hibernation fails to take place, a few or many of the embryos in the eggs in a pod revolve around the lower pole about the twenty-third or twenty-fourth day at 25°C., and hatching begins on or near the thirty-eighth day. Evidence accumulated so far tends to show that this non-hibernating habit may be inherited.

Hibernation in the ordinary Melanoplus differentialis egg can be prevented by exposing eggs under three weeks of age —that is, three weeks at 25°C.—to low temperatures, or, according to O. E. Nelsen (personal communication), it can be broken by subjecting eggs which have already begun to hibernate to cold. After such treatment, when returned to a favorable environment, growth recommences at once and hatching takes place (at 25°C.) with marked regularity about two weeks after revolution.

As was shown by Bodine ('29), the onset of hibernation in these eggs is marked by a striking decrease in the consumption of oxygen and by a cessation of the passage of water into the egg from the external environment.

METHODS AND RESULTS

Eggs containing embryos of known age and with a known temperature history were dropped into B_3 after a small portion of the chorion at the anterior end had been removed so as to permit the more rapid penetration of the fixative. After remaining in the fixative for twenty-four hours or longer, the eggs were opened, dissected under a binocular microscope, and the embryos freed, as far as possible, from yolk granules. They were then washed, dehydrated, and embedded in the usual way. Sections were cut at 5 μ and stained with Heiden-

hain's haematoxylin. Mitotic figures were counted with a Spencer binocular research microscope. Only those cells with distinct, compact chromosomes were included in the count.

In the first series of experiments eggs from a single pod laid by a grasshopper eighteen days previously and kept in an incubator at 25°C. since that time were divided into twelve equal lots. One lot was fixed at 12 o'clock noon and another lot at the end of each successive two-hour interval during the following twenty-four hours. During the night the eggs were kept in a thermos bottle containing water warmed to 25°C.

In the second series of experiments the same procedure was followed with a few changes. Eggs nineteen days old, instead of eighteen, were used and all were kept in a water-bath at 25°C. ± 0.1°, for the entire twenty-four-hour period. This was done because it was thought possible that slight changes in the temperature of the incubator and thermos bottle might account for the variations in the number of dividing cells in the different embryos used in the first series.

In addition, numerous embryos, both actively growing and in the hibernating condition, ranging from those kept seven days at 25°C. to embryos about to hatch, were sectioned and examined. No attempt, however, was made to count dividing cells in these cases.

The results of the two series of experiments are shown in figures 1 and 2. In both instances the number of mitotic figures was found to vary within wide limits. In certain cases, such as 12 o'clock noon in figure 2, the cells in several embryos fixed at the same time were studied. The highest figure found here was almost twice as great as the lowest.

With variations of this sort it is obvious that, in order to obtain results which are statistically valid, it would be necessary to count the dividing cells in very large numbers of embryos. When relatively few embryos are used, as in the present case, the results are not strikingly significant.

When, however, sections of embryos between eighteen and twenty days of age are compared with those from embryos a week older, a point of considerable interest becomes appar-

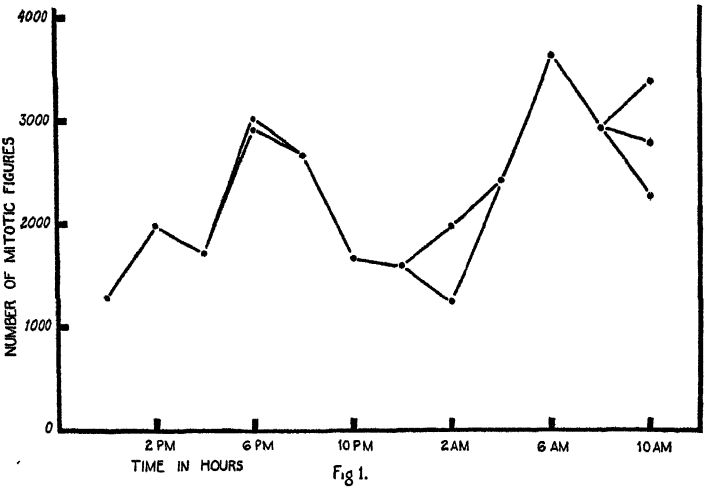

Fig 1.

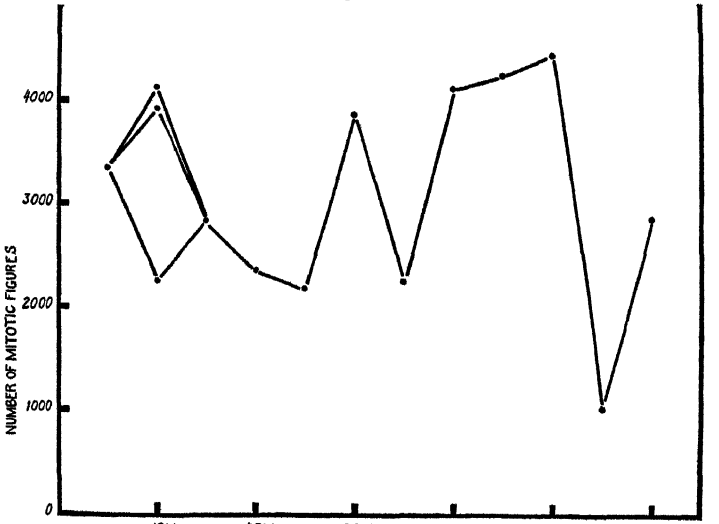

Fig 2.

Fig. 1 Number of mitotic figures in embryos of Melanoplus differentialis fixed at two-hour intervals throughout twenty-four hours. Series 1. For further explanation, see text.

Fig. 2 Number of mitotic figures in embryos of Melanoplus differentialis fixed at two-hour intervals throughout twenty-four hours. Series 2. For further explanation, see text.

ent. As may be seen from the figures, an embryo just under three weeks of age may contain as many as 4400 dividing cells. An embryo a week older contains practically none. Within less than a week the rate of mitotic activity drops from an exceedingly high figure almost to zero. In 1923, Carothers noted, for several other species of grasshopper, that embryos sectioned during hibernation rarely showed dividing cells.

The regularity and decisiveness with which cell division stops in the Melanoplus differentialis egg raises many interesting questions. In what way, for example, does cold alter conditions in the egg so that cell division can be resumed? Is the effect exerted on the yolk so as to change certain foodstuffs—let us say—to a more available form? Or, perhaps, the low temperature changes the permeability of the membranes surrounding the embryo and yolk so that the water needed for further growth can pass into the egg. Or, possibly, the cold may affect the cells of the embryo itself. Then, again, in what way does a long exposure to a moderate temperature compare with a short exposure to cold? The final result—that is, resumed cell division—is the same in both cases. A chemical and physical study of conditions in the egg just before and after the beginning of hibernation and again after exposure to cold seems especially desirable.

In conclusion, the author wishes to thank Dr. C. E. McClung, who suggested the problem and under whose direction it was completed.

SUMMARY

1. An embryo of the grasshopper, Melanoplus differentialis, taken from an egg kept from eighteen to twenty days at 25°C., from the time of laying, may contain as many as 4400 or as few as 1000 dividing cells.

2. Melanoplus differentialis embryos from eggs kept twenty-seven days at 25°C., as well as those older and still in the hibernating condition, contain few or no mitotic figures.

3. No rhythms of mitotic activity correlated with diurnal periods were detected.

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INDEX

(ACRIDIDAE, Orthoptera). Life cycle, sex differentiation, and testis development in Melanoplus differentialis 467
African lungfish, under normal conditions and under conditions of prolonged estivation and recovery. Blood formation in the. 319
AMANO, KAGEYAS W. The labyrinth of Bufo vulgaris Japonicus larva. Especially, upon the morphologic study of the perilymphatic space, in connection with the membranous labyrinth and auditory capsule 207
Aphididae. The evolutional significance of the chromosomes of . . 373
Auditory capsule. The labyrinth of Bufo vulgaris Japonicus larva. Especially, upon the morphologic study of the perilymphatic space, in connection with the membranous labyrinth and 207

BLOOD formation in the African lungfish, under normal conditions and under conditions of prolonged estivation and recovery . 319
Branchial nerve in Mytilus edulis and its relation to the problem of nervous control of ciliary activity. The distribution of the ... 195
BROWN, GEORGE V. E. The morphology of Spironympha, with a description of a new species from Reticulitermes hesperus Banks 291
Bufo vulgaris Japonicus larva. Especially, upon the morphologic study of the perilymphatic space, in connection with the membranous labyrinth and auditory capsule. The labyrinth of 207

CHLAMYDOMONAS. The vacuome of the flagellate 527
Chromosomes of Aphididae. The evolutional significance of the . 373
Chromosome studies. II. Synapsis in the Tettigidae, with special reference to the presynapsis split 119
Ciliary activity of the lamellibranch gill. An investigation of the nervous system as a possible factor in the regulation of 147

DAS, B. K. Observations on the 'renal-portal' perfusion in etherized birds 309
Desmognathus fusca spermatogenesis. Notes on the postnuclear, acrosome-seat granules, and 'vacuome' in 597
Drosophila melanogaster Meigen and Popillia japonica Newm. Recovery from immersion in water. An index of metabolism and the condition of the gonads in 435

EDDY, MILTON WALKER. Recovery from immersion in water. An index of metabolism and the condition of the gonads in Drosophila melanogaster Meigen and Popillia japonica Newm. . . . 435
Evolutional significance of the chromosomes of Aphididae. The . 373

FLAGELLATE Chlamydomonas. The vacuome of the 527

GATENBY, J. BRONTË. Notes on the postnuclear, acrosome-seat granules, and 'vacuome' in Desmognathus fusca spermatogenesis 597
Germ cells in Sphaerium striatinum (Lam.). History of the .. 545
Gonads in Drosophila melanogaster Meigen and Popillia japonica Newm. Recovery from immersion in water. An index of metabolism and the condition of the .. 435
Grasshopper embryo. Insect development. II. Mitotic activity in the 613

HAGAN, HAROLD R. The embryogeny of the polyctenid, Hesperoctenes fumarius Westwood, with reference to viviparity in insects 1
HALL, RICHARD P., AND ROSS F. NIGRELLI. The vacuome of the flagellate Chlamydomonas 527
Hesperoctenes fumarius Westwood, with reference to viviparity in insects. The embryogeny of the polyctenid, ... 1
HICKMAN, CLEVELAND P. The spermiogenesis of Succinea ovalis Say, with special reference to the components of the sperm 243

JORDAN, H. E., AND C. C. SPEIDEL. Blood formation in the African lungfish, under normal conditions and under conditions of prolonged estivation and recovery .. 319

LABYRINTH of Bufo vulgaris Japonicus larva. Especially, upon the morphologic study of the perilymphatic space, in connection with the membranous labyrinth and auditory capsule. The .. 207
Lamellibranch gill. An investigation of the nervous system as a possible factor in the regulation of ciliary activity of the . . . 147
Life cycle, sex differentiation, and testis development in Melanoplus differentialis (Acrididae, Orthoptera) 467
LUCAS, ALFRED M. An investigation of the nervous system as a possible factor in the regulation of ciliary activity of the lamellibranch gill 147

LUCAS, ALFRED M. The distribution of the branchial nerve in Mytilus edulis and its relation to the problem of nervous control of ciliary activity 195

MELANOPLUS differentialis (Acrididae, Orthoptera). Life cycle, sex differentiation, and testis development in 467
Membranous labyrinth and auditory capsule. The labyrinth of Bufo vulgaris Japonicus larva. Especially, upon the morphologic study of the perilymphatic space, in connection with the 207
Metabolism and the condition of the gonads in Drosophila melanogaster Meigen and Popillia japonica Newm. Recovery from immersion in water. An index of 435
Mitotic activity in the grasshopper embryo. Insect development. II. 613
Mytilus edulis and its relation to the problem of nervous control of ciliary activity. The distribution of the branchial nerve in 195

NELSEN, OLIN E. Life cycle, sex differentiation, and testis development in Melanoplus differentialis (Acrididae, Orthoptera) 467
NIGRELLI, ROSS F. See Richard P. Hall 527

ORTHOPTERA). Life cycle, sex differentiation, and testis development in Melanoplus differentialis (Acrididae, ... 467

PERILYMPHATIC space, in connection with the membranous labyrinth and auditory capsule. The labyrinth of Bufo vulgaris Japonicus larva. Especially, upon the morphologic study of the 207
Polyctenid, Hesperoctenes fumarius Westwood, with reference to viviparity in insects. The embryogeny of the 1
Popillia japonica Newm. Recovery from immersion in water. An index of metabolism and the condition of the gonads in Drosophila melanogaster Meigen and 435

'RENAL-PORTAL' perfusion in etherized birds. Observations on the 309
Reticulitermes hesperus Banks. The morphology of Spironympha, with a description of a new species from 291
ROBERTSON, W. R. B. Chromosome studies. II. Synapsis in the Tettigidae, with special reference to the presynapsis split 119

SEX differentiation, and testis development in Melanoplus differentialis (Acrididae, Orthoptera). Life cycle, ... 467
SHINJI, ORIHAY. The evolutional significance of the chromosomes of Aphididae 373
SLIFER, ELEANOR H. Insect development. II. Mitotic activity in the grasshopper embryo 613
Sphaerium striatinum (Lam.). History of the germ cells in 545
SPEIDEL, C. C. See H. E. Jordan 319
Spermatogenesis. Notes on the postnuclear, acrosome-seat granules, and 'vacuome' in Desmognathus fusca 597
Spermiogenesis of Succinea ovalis Say, with special reference to the components of the sperm. The 243
Spironympha, with a description of a new species from Reticulitermes hesperus Banks. The morphology of 291
Succinea ovalis Say, with special reference to the components of the sperm. The spermiogenesis of 243
Synapsis in the Tettigidae, with special reference to the presynapsis split. Chromosome studies. II. 119

TESTIS development in Melanoplus differentialis (Acrididae, Orthoptera). Life cycle, sex differentiation, and 467
Tettigidae, with special reference to the presynapsis split. Chromosome studies. II. Synapsis in the 119

'VACUOME' in Desmognathus fusca spermatogenesis. Notes on the postnuclear, acrosome-seat granules, and 597
Vacuome of the flagellate Chlamydomonas. The 527
Viviparity in insects. The embryogeny of the polyctenid, Hesperoctenes fumarius Westwood, with reference to 1

WOODS, FARRIS H. History of the germ cells in Sphaerium striatinum (Lam.) 545

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